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Youhe Gao *Editor*

Urine Proteomics in Kidney Disease Biomarker Discovery

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Preface

I never thought about writing or editing a book since it is boring and not very useful. Until one day, Mandy from Springer came to my little shared office asking me if I wanted to edit a book. My neighbor lab, PI Hongbing thought I might have something to write about and recommended me to her. It was the time when we had just developed in the lab a way to save urinary proteins on the membrane cheaply and simply. It was named urimem. I was thinking about people who will all have biological samples in their medical record. This may potentially change the gear for biomarker development. If medical records can change the face of medicine, adding biological samples to the information already in the medical record would very likely change the face of medicine again. My excitement did not last very long when I realized that saving urine may not be necessary for all people. It may only be useful for urinary disease patients.

I sat in my cube wondering why people need this urimem in their medical records. In my mind, biomarkers were generated in organs and traveled to the blood waiting for detection, then the blood was full of biomarkers and they jammed the circulation. I realized that people cannot survive if the biomarkers stay in blood forever. I recalled what I taught in angiogenesis class. Angiostatin needs to be continuously infused to maintain the effective concentration in blood. The things that enter the blood need to be removed. The blood needs to be stable. Biomarkers from other organs need to be removed too. Where are they supposed to go? Urine is very likely. I started searching the Internet for teaching material on homeostasis. From a PPT I am still showing around, it showed that in order to maintain the homeostasis of internal environment, wastes were removed by kidney, liver, lung, and skin. Urine is the most directly connected to blood, the cleanest, most accessible, completely noninvasive body fluid.

If human beings were designed and created, urine was probably set just for us to remove the wastes and check the status of our bodies.

The book as a form of publication is slow compared to papers, not to mention meeting abstracts and personal blogs. If a book needs to be edited and published, it has to be usable for a while, at least for a few years. What information is useful for a few years for researchers and students in the field? The most up-to-date

development in the field is not that durable. The most durable things are the things that need to be considered when people want to design an experiment in the field. I believe that nobody needs a book of "the current affair." Before the book is on the shelf or even online, the information is not up-to-date anymore. We need a book of "all things considered." The chapters chosen to propose an idea need to be considered. Most of them are not reviews. Some of the chapters are almost the reformat of the paper.

I have been working in the field for a number of years. But I know I am not well known, let alone famous. It is hard to edit a book without supporting participants. I thank those authors who agreed with me on the way this book is organized. I thank all the authors who spent their time to contribute. I thank all my former and current lab members who contributed to the book. I enjoyed working with you. I enjoyed the weekly discussion at the lab meeting. I thank my collaborators Mingxi, Zhihong, and Xiangmei for giving me the chance to work and discuss together. I thank Peng from Springer for going through the tedious editing process. I thank my colleague Xianda and Chengyu for their support. I thank my parents for giving me life and freedom. I thank my wife and other family members who let me enjoy science.

I know this book is far from what I planned and expected. Lots of related topics are not covered because of my limited knowledge and ability, and because of the availability of potential authors. I hope there will be more people participating if we have the next edition in a few years. I believe in this field, which has the potential to change the face of biomarker, biobanking, biotechnology, and eventually, medicine and health.

Urine smells good.

It tastes even better.

NJ, USA, July 2014

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Part I
Urine as Biomarker Source

Chapter 1
Urine Is a Better Biomarker Source Than Blood Especially for Kidney Diseases

Youhe Gao

Abstract Change is the soul of biomarker definition. Changes are more likely to be removed from blood because of homeostasis mechanisms of the body. Therefore, urine is probably a better biomarker source than blood. The road map to the urinary biomarker era is proposed. Researchers are reminded the potential opportunities and risks in their study design. Kidney diseases are emphasized as they produce most significant changes in urine.

Keywords Change · Homeostasis · Confounding factors · Animal model

1.1 Urine Is a Better Biomarker Source Than Blood

In 1998, biomarker was defined by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. It was also defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [2]. From Wikipedia, "a biomarker is a measurable characteristic that reflects the severity or presence of some disease state. More generally a biomarker is anything that can be used as an indicator of a particular disease state or some other physiological state of an organism" [3].

They all emphasized that a biomarker relates to a condition, a biomarker has to be measurable, and a biomarker can be anything. When the biomarker discovery process was analyzed, we can see there are always at least two groups to compare in the study.

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The results are always the differences of the two groups. Should we say the most fundamental nature of the biomarker is "change" from one state to another state, most commonly a diseased state from healthy state [4]?

Knowing its nature helps us to trace where it goes and where to find it. Since blood connects to all the important organs, it collects all the changes from the body. It is obvious we should all look for biomarker in blood. And it is accessible with almost no harm. We have been doing that for decades. It seems the most agreed consensus of researchers in biomarker field. The question is how long does a change can stay in blood. It depends on how fast the biomarker is produced and arrives blood and how fast it leaves the blood. In healthy state, all the cells enjoy their bath in the internal environment. They do not like any changes. The reason we survive until now is all because the body develops the most important homeostasis mechanisms.

Homeostasis is the property of a system in which changes are removed by negative mechanisms so that internal conditions remain stable or relatively constant. The concept was described by Claude Bernard in 1865 and the word was coined by Walter Bradford Cannon in 1926 [5].

With these negative feedback homeostatic mechanisms of the body, the change from the organs tends to be removed from the internal environment, mainly blood, to external environments as fast as the body can, via liver, kidney, lung, and skin. The change eventually goes to the outside via bile, urine, breath, and sweat. At the outside environment, the change encounters no mechanism to remove it, even though it may continue to degrade.

What if there is a new homeostatic point for the disease in the blood? Will change be easier to detect in the blood than in urine? For certain chronic conditions, the body will work at a changed but rather stable condition for a period of time. But before that new homeostatic point is reached, the homeostatic mechanisms tend to remove changes when they were triggered by the change. The new homeostatic point can be considered as an uncompensatable state compare to the previous healthy point, even all the negative mechanisms of homeostasis were all applied. If this is the case, the most sensitive changes happen before the new homeostatic point is ever reached. And the first change should be the one that was removed from the blood to the outside environment via various mechanisms. In other word, the most sensitive changes should be detected earliest in the discharge of the body rather than the most basic functional component of the body which is blood. In biomarker study, the earliest and the most sensitive ones are better biomarkers. In this sense, the best biomarkers are not in the blood. The biomarkers that were found in the blood were merely the uncompensated changes at a rather later stage of a relatively stable condition. Better biomarkers can be expected in other discharges, especially urine. Blood is a good place to find biomarker as this biomarker stays long in blood and we are fast enough to catch it in time. Antibody type of biomarkers and some long half-life proteins in blood are probably the case. We may miss it if it leaves blood fast. But if we wait at the outside to check the bile, urine, breath, or sweat, we will definitely find the remains of the change, as long as we collect samples continuously, unless the remains completely lose its special characteristics. Even though it may lose its characteristics, it may still change the quantity of some uncharacteristic molecules.

Urine is probably the best place to find the change since itself is the filtrate of blood and contains all the soluble biomarkers. Bile is hard to collect as it mixes with stool. Sweat is hard to collect because of its trace amount. Breath is a good place for volatile biomarkers since it can be collected continuously and non-invasively. Preservation methods of the breath samples will be developed if the sample can be proved to be valuable for biomarker research.

What are the things that can potentially present in urine as biomarker? It is well known that small molecules are abundant [6]. MicroRNAs are identified in urine too. Of course, there are also thousands of different kinds of proteins in urine [7, 8]. The fact has not been fully acknowledged to clinicians. Quite numbers of doctors still believe that proteins only appear in urine in some pathological conditions. It is actually good to have only trace amount of proteins in healthy state, but also has the potential to have abundant amount of protein when in disease state. In other words, urine can accumulate and tolerate huge changes without harming the body, which is the best feature of being the best biomarker source. People may still argue there is probably a different homeostasis state of the blood in disease condition, which give us a long time window for biomarker identification. It is possible to have many different homeostasis states of blood for many different conditions. But the differences between the disease condition and healthy condition should not be big. Cells in the body cannot tolerate big differences. And if you count the main component of the blood, the differences are only a small percentage change. But if some of these differences pass to urine, compare to the main component of urine, the differences would be a big percentage change. Big change means good biomarker.

Is it true that changes in blood can be magnified in urine? Let us make a change in blood. The change should be able to change the function of blood, let us look at the component changes in the blood and the urine in the same system by the same detection method. Two anticoagulants heparin and argatrabon were used to change blood coagulation status of adult female SD rats. Plasma and urine protein composition in six SD female rats before and after treatment was analyzed. With the exactly same LC-MS/MS method, much more differences can be identified in urine than in plasma. Those changed proteins in urine showed no significant changes in corresponding blood of the same animal [9].

Not many biomarker researchers work on both plasma and urine in one study. But there were a few. In 2009, Payne et al. found that "in all negative class comparisons and for all biomarkers, measurement of the biomarkers in urine DNA was more sensitive than for plasma DNA" (Table 1.1) [10]. In 2013, Wu et al. showed in the result that "Urinary Angiostatin is Able to Discriminate Active SLE from Inactive SLE" and "Urinary Angiostatin Positively Correlates with Lupus Disease Severity". The same result cannot be achieved in serum (Fig. 1.1) [11]. Even lung disease can show more sensitive biomarker in urine than in blood. Huang et al. showed with an exacerbation of chronic obstructive pulmonary disease (COPD) compare to blood desmosine, urine desmosine provided better separation between healthy and diseased group (Table 1.2) [12]. In blood, the desmosine level ranged at 0.12–0.23 ng/ml for healthy control, while for exacerbated COPD, patients' blood desmosine ranged at 0.21–0.37 ng/ml. There was an overlap which

Table 1.1 Frequency of aberrant methylation in urine and plasma DNA [12]

	GSTP1		RASSF2	
	Urine (%)	Plasma (%)	Urine (%)	Plasma (%)
Positives (%)				
Young asymptomatic males	6	20	37	2
Biopsy negative	59	31	82	16
All stages PCa	81	39	59	31
T1 (n = 47 U, 46 P)	83	37	96	35
T2 (n = 28 U, 25 P)	71	32	82	16
T3 (n = 7)	100	71	100	57
T4 (n = 2)	100	50	100	50
Median DNA (range), ng/ml				
Young asymptomatic males	0 (0–0.07)	0 (0–0.00[a])	0 (0–0.09)	0 (0–0.00[a])
Biopsy negative	0.001 (0–0.15)	0 (0–0.02)	0.007 (0–0.70)	0 (0–0.04)
All stages PCa	0.008 (0–91.18)	0 (0–0.27)	0.025 (0–112.45)	0 (0–0.19)
T1 (n = 47 U, 46 P)	0.006 (0–91.80)	0 (0–0.18)	0.024 (0–112.45)	0 (0–0.19)
T2 (n = 28 U, 25 P)	0.008 (0–0.88)	0 (0–0.05)	0.022 (0–0.91)	0 (0–0.00[a])
T3 (n = 7)	0.029 (0.001–14.37)	0.001 (0–0.27)	0.042 (0–19.08)	0.0005 (0–0.02)
T4 (n = 2)	n.a. (0.014–0.14))	n.a. (0–0.00[a])	n.a. (0.07–0.08)	n.a. (0–0.00[a])
	HIST1H4K		TFAP2E	
	Urine (%)	Plasma (%)	Urine (%)	Plasma (%)
Positives (%)				
Young asymptomatic males	14	8	82	2
Biopsy negative	84	31	100	16
All stages PCa	92	31	100	18
T1 (n = 47 U, 46 P)	96	28	100	24

(continued)

Table 1.1 (continued)

	HIST1H4K		TFAP2E	
	Urine (%)	Plasma (%)	Urine (%)	Plasma (%)
T2 (n = 28 U, 25 P)	82	28	100	4
T3 (n = 7)	100	71	100	29
T4 (n = 2)	100	0	100	0
Median DNA (range), ng/ml				
Young asymptomatic males	0 (0–0.02)	0 (0–0.01)	0 (0–0.24)	0.013 (0–0.00[a])
Biopsy negative	0.004 (0–0.16)	0 (0–0.00[a])	0.052 (0.001–0.94)	0 (0–0.00[a])
All stages PCa	0.008 (0–47.94)	0 (0–0.18)	0.096 (0.004–27.80)	0 (0–0.14)
T1 (n = 47 U, 46 P)	0.008 (0–47.94)	0 (0–0.18)	0.106 (0.01–27.80)	0 (0–0.14)
T2 (n = 28 U, 25 P)	0.008 (0–0.72)	0 (0–0.01)	0.096 (0.01–4.42)	0 (0–0.00[a])
T3 (n = 7)	0.024 (0.00–4.04)	0.007 (0–0.02)	0.377 (0.01–6.81)	0 (0–0.01)
T4 (n = 2)	0 (0.01–0.06)	0	n.a. (0.08–0.09)	0

[a] Indicates positive at >0.0001 ng/ml

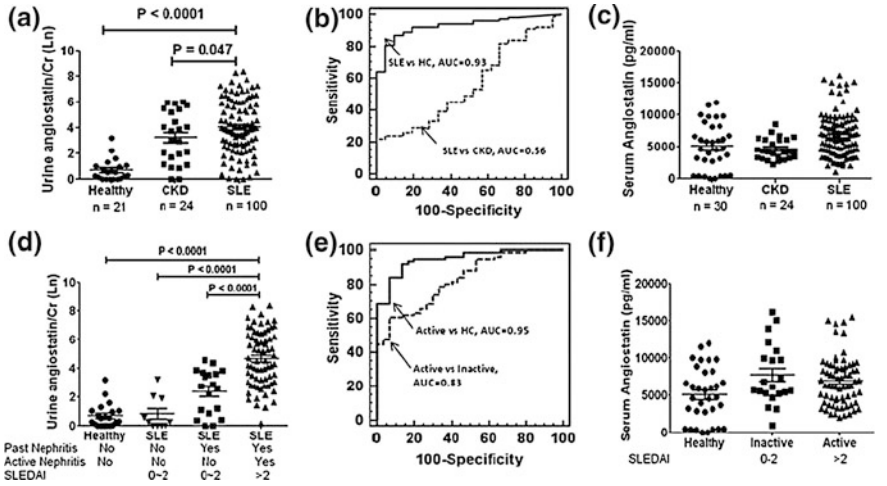

Fig. 1.1 Validation of urinary angiostatin as a marker in a larger independent cohort of SLE patients ($n = 100$), chronic kidney disease (*CKD*) patients ($n = 24$), and healthy controls ($n = 21$). **a** Urinary angiostatin levels as determined by ELISA are expressed as the natural logarithm of the absolute values of urinary angiostatin (pg/ml) normalized against urine creatinine levels. **b** ROC curve analysis was performed and the area under the curve (*AUC*) was used to assess the sensitivity and specificity of urinary angiostatin in discriminating SLE from healthy controls or CKD controls. **c** Serum angiostatin levels were measured in samples from the same subjects shown in (**a**) and (**b**), with SLE patients ($n = 100$), CKD controls ($n = 24$), and healthy controls ($n = 30$) using ELISA. **d** SLE patients were divided into inactive and active groups according to SLEDAI and renal SLEDAI values. Inactive SLE: SLEDAI = 0–2, rSLEDAI = 0; active SLE: SLEDAI > 2, rSLEDAI > 0. Urinary angiostatin levels as determined by ELISA are expressed as the natural log of absolute values of urinary angiostatin (pg/ml) normalized against urine creatinine levels. **e** The sensitivity and specificity of urinary angiostatin in discriminating active SLE from inactive SLE or healthy controls were assessed using the AUC in a ROC curve analysis. **f** Serum angiostatin levels were also measured in the same SLE patients described above [11]

compromises the usage of this marker. But in urine, for healthy control, desmosine ranged at 6–10 ng/mg creatinine; and for diseased group, it ranged at 14–22. These two ranges were very well separated. It makes the urine desmosine a much better biomarker. In 2008, Smith et al. from Harvard Medical School for the first time identified urinary biomarkers that predict the presence of brain tumors [13]. It is amazing that these biomarkers could travel from brain to urine. Even though these studies were not in large scale, they provided useful clues for us. I limited myself to protein markers, but urine does not limit its potential to proteins markers only. Small molecules, microRNA and DNAs, can all be present in urine.

These results suggest that urine biomarker should be taken more seriously. To take advantage of this conceptual change, we should summarize all previously suggested biomarker clues found in blood and check them all out in urine again, no matter they were validated or not in blood. Some good biomarkers in blood may perform even better in urine. Some not so good biomarkers in blood may be acceptable ones in urine. New intellectual properties will be generated. More funding, more researchers

Table 1.2 Demographic and desmosine data for group 2 consisting of healthy volunteers and patients with an exacerbation of chronic obstructive pulmonary disease (COPD) [13]

Sample type	Group 2			
	Urine and sputum		Blood	
Group	Healthy volunteers (HV2a)	Patients with "during an exacerbation" COPD	Healthy volunteers (HV2b)	Patients with "during an exacerbation" COPD
Number of participants	62	50[a]	19	102[a]
Gender (M/F)	24/38	24/26	18/1	43/59
Smoking status (smokers/E-smokers/ non-smokers/ unknown)	13/41/8/0	31/2/15/2	10/0/9/0	55/33/0/14
Age (years)	22 (21–45)	69 (60–74)[b]	68 (65–73)	72 (66–79)
Body mass index	25 ± 4	26 ± 7	NA	26 ± 7
FEV_1 (% predicted)	103 ± 13	39 ± 16[b]	NA	47 ± 18
uDES (ng/mg creatinine)	8 (6–10)	16 (14–22)[b]	–	–
bDES (ng/ml)	–	–	0.17 (0.12–0.23)	0.30 (0.21–0.37)[b]

Data are shown as median (IQR) or mean ± SD
Note that the healthy volunteers recruited for urine and sputum analysis (HV2a) were different from those for blood analysis (HV2b)
[a] A total of 47 patients with during an exacerbation COPD were the same as those who had urine, sputum and blood collected
[b] $p < 0.001$, versus healthy volunteers, Mann–Whitney test
bDES, blood desmosine; FEV_1, forced expiratory volume in 1 s; uDES, urinary desmosine

and more companies should start to work on this opportunity. It has the potential to change the face of medicine.

The potential of urinary biomarker has not realized in the biomarker field. When searching the PubMed with urine and biomarker, the number of publications is less than 10 % of that searching with (blood or serum or plasma) and biomarker. This is already an overestimation of the studies in urine, since even if there was word urine in the paper, the paper was counted as biomarker study in urine. There is a manually curated urinary protein biomarker database in the laboratory (http://122.70.220.102/biomarker/index.asp) [14], which covers all urinary protein biomarker studies in both human and animals we can find. Peptides and small molecules were not included yet because of the limited manpower. Up to the time this chapter was written, there were about 500 papers, only a small fraction compare to the biomarker studies in blood, which was about 300,000 papers, accumulated these years.

In terms of biomarker source, accessible non-invasively, low background, relatively stable liquid, connected to blood, and potential to accept all kinds of changes are the best features we can ask for. Personally I cannot foresee any better biomarker source than urine in human being, mutating at current rate.

1.2 Kidney Disease Biomarkers Are the Breakthrough Point in Biomarker Research

I think urine will be a better biomarker source than blood for disease from many or even all organs. But it is probably best for diseases of urinary system. Not many closer relationships between a freely accessible body fluid and a vital organ than between urine and kidney exist. Saliva and salivary glands, sweat and sweat gland are probably other examples.

Obviously functional changes in kidney can induce massive changes in urine. We may not be able to tell which changes are for which disease condition specifically so far. But we have to admit the huge changes are there to study, which is better than looking for changes where changes are not supposed to be big in that massive background.

There is not so obvious feature of kidney. It is the organ that connects with two most important easily accessible body fluids, blood and urine. This feature provides us a unique opportunity to observe its functional changes by looking at and comparing its input and output without touching the kidney itself [15].

1.3 What's the Next Step?

There are a few hundreds urine biomarker studies. If the place was right, why did not we harvest many usable biomarkers? What could be the major problem in urinary biomarker studies? Urine is very different from blood. When studies were done in blood, the first problem was the major components are too much, and the changes (biomarkers) were only a small percentage. It requires very sensitive detection methods to see the small changes. The second problem was that the biomarkers were changing with time. The speed of changing depends on the speed it is produced and the speed it is removed. Only the biomarkers that were big changes and stayed in blood for a long time could be detected. This makes the biomarker discovery in blood difficult. When the studies were done in urine, the major problem was there are too many factors that had effects on urine. The advantage is we can see changes in urine when there are only a little physiological or pathophysiological changes. The disadvantage is too many changes are intertwined and it is hard to differentiate which factor causes which changes.

There are two ways to tackle the problem.

One is to figure out the effects by changing one factor at a time [16, 17]. This is probably can be done in animals easier than in human. As for factors in healthy people, they are probably still countable. But it will still take us quite some time to figure each one out.

The other way is to save a lot of samples and analyze a lot of samples to generate big data. But analyzing the big data, we eventually will figure out the associations between each factor and its effect in urine. Urine is hard to save because it is much

diluted and takes a lot of space. We have to remove the water part of the urine to make it taking less space. By filtering it through membrane (nitrocellulose or certain PVDF), proteins can bind to the membrane. We can dry the proteins on membrane and keep the membrane in a vacuum bag. We named it Urimem. In this condition, the enzymes are inactive, nothing can grow. We may store the samples even at room temperature for quite a long time. It is simple, economical and environmental friendly as it does not require a lot of organic solvent to precipitate protein. It makes saving large amount of clinical samples possible [18]. I propose that starting from now, we should save all the urine samples from the patients before their kidney biopsy. One day, we would be able to compare urine analysis and biopsy result. It is not impossible that we eventually replace kidney biopsy with urine analysis. With these samples, biomarker studies of other diseases can be sped up too. We can afford even prospective studies with real biological samples instead of survey data only. If urine can be proved to the gold mine, we may see saving everybody's urine sample possible and meaningful. Keeping medical record changed the face of medicine for the last one hundred years [19]. May we change the face of medicine for the next one hundred years by adding biological samples for all the patients (or even healthy people) to the current information-only medical records?

The imminent question is what the physiological variations are in human urine. With limited ability, we tried to analyze a few people's urine proteomes [20]. We proposed that if the stable proteins in healthy urine were changed in a patho-physiological condition, these proteins are more likely to be good biomarkers. That study was a conceptual preliminary experiment. More effort has been made in that direction [21, 22] even though we are still far from knowing the normal variation of the human urine proteome.

1.4 Opportunities and Risks

There will be great opportunities that anybody in the biomarker field does not want to miss. There are huge amount of clues accumulated in 300,000 papers in the past few decades for biomarkers in blood. Only a very small fraction of those papers had the word "urine" in them, which implies that those biomarkers have probably never been tested in urine. Researchers and/or companies in biomarker field may easily take advantage of the free information and try to validate them in urine. New intellectual properties can be produced if any of the biomarkers works better in urine. There are great chances of finding a considerable numbers of new biomarkers in a rather short period of time [23]. This may nurture many new biomarker companies in the biotechnology field.

Biomarker researchers who insist on working only in blood may face great risks of losing the value of their findings in blood, if somebody else validates them in urine independently. Although there are blood-only biomarkers, having a comprehensive validation protocol will help eliminate any possible loopholes [23].

There are great opportunities and risks in the coming urine biomarker era.

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Chapter 2
Urine Reflection of Changes in Blood

Menglin Li

Abstract The most important of nature of biomarker is changes. Blood is under strict homeostatic control which means changes tend to be removed from blood. Urine is a partial filtrate of blood, reflects systemic physiology but with no homeostatic mechanism. However, changes induced directly into the blood can be more sensitively detected in urine than in blood itself. This indicates that urine may serve as a source for more sensitive detection of protein biomarkers than blood.

Keywords Change · Biomarker · Urine · Blood

Theoretically, urine may be a better source for biomarker discovery than plasma. Given that change is an essential property of a biomarker, the strict homeostatic regulation of plasma composition would in theory act to remove any changes in blood. While urine collects all wastes from the body, it can accumulate more and bigger changes [1]. Therefore, urine serves not only as an ideal source of biomarker discovery for diseases of the kidney and other tissues of the urogenital system but also as a potential source of information on diseases in other physiological systems.

In comparison to plasma, urine has many other advantages as a source for biomarker discovery. First, urine can be collected noninvasively, frequently, and in large quantities. Second, while plasma is generally obtained at a single time point, multiple urine samples can be collected over a period of time, allowing for an easier monitoring of time-dependent changes in biomarker levels. Third, proteolytic degradation may be complete prior to collection of urine [2] and because proteases are activated during blood collection [3], the urinary proteome shows much greater stability compared with that of plasma. Urinary proteins, for example, show no significant changes when urine is stored for three days at 4 °C or for 6 h at room

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temperature [4]. The use of membranes to store urinary proteins is simple and economical and facilitates biomarker research by making large-scale storage of clinical urinary samples possible [4].

In paper published on Science China Life Science [5], two different anticoagulants were used (unfractionated heparin and argatroban) to disrupt the blood coagulation status of adult female SD rats. Changes in urine and plasma proteomic profiles in response to treatment with the drugs were compared with exactly same standard to observe whether there are more changes in urine.

Six adult female Sprague-Dawley rats were initially anesthetized by an intravenous injection of 20 mg kg^{-1} pentobarbital sodium, after which a catheter was inserted for urine sampling.

Saline (0.9 % NaCl in sterile water) was first administered via the great saphenous vein as a single bolus with a volume equivalent to that of the anticoagulants. Urine was collected over the following 30 min as a control specimen. Rats then received a bolus of argatroban (2 mg kg^{-1}) the same vein, after which urine was collected over the following 30 min. Only rats that showed no gross hematuria were considered to be successful preparations and proceeded to the next stage. Urine was centrifuged at 3,000 g for 10 min at 4 °C. After removal of precipitates, urinary proteins were extracted by acetone precipitation [6] and subjected to quantitation by the Bradford method.

Venous blood was collected by aspiration from the femoral vein at three time points: 5 min after saline injection and 5 min and 30 min after administration of anticoagulants. For the assessment of clotting time (CT), 25 μL of the blood sample was used. The remaining blood sample was immediately transferred to a plastic tube containing 0.109 M sodium citrate at a ratio of 1:9, gently mixed and centrifuged at 2,000 g at 4 °C for 10 min to obtain citrated plasma. Plasma was stored at −80 °C until further analysis.

Heparin (5 mg/kg) was given intravenously to another group of rats by the same procedure as argatroban experiment described above.

The identical proteomic analysis approach was used to analyze the protein composition of rat urine and plasma samples before and after treatment with anticoagulants. Software Progenesis were used to perform label-free quantification. Briefly, for protein identification, the false positive rate was 1 %. For quantification, only unique peptides and proteins with at least two peptides were included. Besides, the direction of change in each animal should be consistent and the fold change should be ≥1.5 in at least one animal.

LC-MS/MS analysis detected consistent heparin-induced changes in the levels of 27 proteins in urine but only three proteins in plasma (Table 2.1) in three drug-treated rats and three control rats. There was no overlap between these groups of proteins. Consistent changes in the levels of 61 proteins were detected in urine in response to treatment with argatroban, only one of which changed in plasma. The number of proteins identified in plasma was much smaller than the number detected in urine. These results indicate that changes in protein levels may be more sensitively detected using LC-MS/MS in urine than in plasma.

Table 2.1 Changed proteins in heparin-treated group [5]

Accession	Max fold change in urine	Change tendency in urine	Max fold change in plasma	Change tendency in plasma	Description
ITIH3_RAT	2.5	up	N		Inter-alpha-trypsin inhibitor heavy chain H3
CO4_RAT	2.8	up	N		Complement C4
IGG2C_RAT	3.1	up	N		Ig gamma-2C chain C region
FIBB_RAT	2.1	up	N		Fibrinogen beta chain
HEMO_RAT	2.6	up	N		Hemopexin
FIBG_RAT	3.4	up	N		Fibrinogen gamma chain
AFAM_RAT	3.7	up	N		Afamin
APOA1_RAT	3.4	up	N		Apolipoprotein A-I
FETUB_RAT	2.9	up	N		Fetuin-B
CADH1_RAT	1.5	down	N		Cadherin-1
ALBU_RAT	3.1	up	N		Serum albumin
FETUA_RAT	2.9	up	N		Alpha-2-HS-glycoprotein
APOH_RAT	2.4	up	N		Beta-2-glycoprotein 1
EGF_RAT	2.3	down	N		Pro-epidermal growth factor
FIBA_RAT	2.4	up	N		Fibrinogen alpha chain
TRFE_RAT	2.5	up	N		Serotransferrin
CO3_RAT	2.7	up	N		Complement C3
UROK_RAT	3.5	down	N		Urokinase-type plasminogen activator
DNAS1_RAT	2.3	down	N		Deoxyribonuclease-1
IL4RA_RAT	1.8	down	N		Interleukin-4 receptor subunit alpha
KLK7_RAT	2.0	down	N		Glandular kallikrein-7
SPA3 N_RAT	2.3	up	N		Serine protease inhibitor A3N
KLK1_RAT	2.1	down	N		Kallikrein-1
APOA4_RAT	3.3	up	N		Apolipoprotein A-IV
MEP1A_RAT	5.1	down	N		Meprin A subunit alpha
PLMN_RAT	3.7	up	N		Plasminogen
6PGL_RAT	2.5	down	N		6-phosphogluconolactonase
IC1_RAT	N		1.6	down	Plasma protease C1 inhibitor
HBA_RAT	N		4.1	up	Hemoglobin subunit alpha-1/2
HBB1_RAT	N		3.9	up	Hemoglobin subunit beta-1

N indicates that the protein was not detected in the sample or failed to fulfill the quantitation criteria

Functional analysis of these differential proteins was performed by the Ingenuity Pathway Analysis (IPA) tool (http://www.ingenuity.com/, Ingenuity Systems, Redwood City, CA, USA). This analysis was focused on the pathways and disease mechanisms in which the proteins are expected to be involved.

The differential proteins of anticoagulants-treated rats take part in acute phase response signaling, LXR/RXR activation, coagulation system intrinsic prothrombin activation pathway and extrinsic prothrombin activation pathway. The proteins involved in coagulation pathway included F2, FGA, FGG, FGB, KLKB1, PLAU, PLG, and SERPINA 1.

Additional six animals were treated with anticoagulants, three for each, to validate LC-MS/MS analysis of changes in the plasma and urine by Western blot. Transferrin and hemopexin were selected for validation because of the abundances and availability of antibodies. In accordance with changes in the levels of these proteins observed via the LC-MS/MS analysis, changes in the levels of both proteins in response to anticoagulant treatment were observed in urine but not in plasma (Fig. 2.1).

Biological fluids such as blood, urine, and CSF serve as common sources of biomarkers, of which plasma is most routinely used [7]. Although plasma perfuses all tissues of the body and theoretically it can collect all the information of disease biomarkers, the role of homeostatic mechanisms in eliminating changes in these biomarkers should not be ignored. Changes in blood biomarker levels are unlikely to persist long enough for detection because they are disturbances of homeostasis.

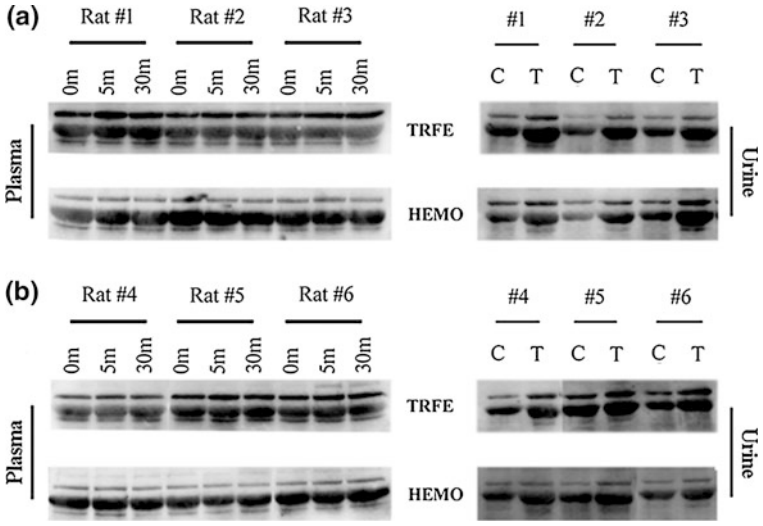

Fig. 2.1 Western blot analysis of transferrin and hemopexin in urine and plasma. 20 μg/lane of urine or plasma proteins was loaded into gels and analyzed using Western blot. Changes in the levels of transferrin were comparable with those detected by LC-MS/MS analysis. **a** heparin-treated group. **b** argatroban-treated group. *C*, before anticoagulants treated; *T*, after anticoagulants treated [5]

Because homeostasis helps to eliminate or excrete changes in biomarker levels into urine, exhaled air, bile, and perspiration, these places may serve potential sources for biomarker discovery.

But there are exceptions. For instance, it is better to detect biomarkers in plasma when they are continuously secreted into blood or have a long half-life. Moreover, biomarkers for acute illnesses such as acute necrotizing pancreatitis (ANP) and acute myocardial infarction (AMI) are excreted into blood in large amounts in a short period of time. Plasma analysis may be more helpful to monitor progression of diseases.

There are several possible reasons that more proteins could be identified in urine than in plasma.

Firstly, any change that is introduced into the blood either internally or externally tends to be cleared by the liver, kidney, and/or other organs via a variety of mechanisms in order to maintain the homeostasis of the blood. In contrast, urine is the place that most of the wastes in blood are dumped into, and thus tolerates changes to a much higher degree. Biomarkers are the measurable changes associated with a physiological or pathophysiological process. Therefore, they are more likely to be magnified and detectable in urine than their counterparts in blood.

Secondly, for proteomics and protein biomarker experiments, high dynamic range of protein concentrations in human plasma leads to a tremendous analytical challenge. Plasma proteins have a high dynamic range, spanning at least 10^9 [8]. Albumin constitutes about half of the plasma proteins. In contrast, many potential biomarkers in plasma are at very low abundance. High-abundance proteins limit the identification of low-abundance proteins by LC-MS/MS.

Thirdly, urine as a result of being a filtrate of plasma is relatively simple in composition, the dynamic range of urinary proteins is about 10^6 [9]. Accumulated changes in urine composition are not likely to be masked, and some molecular species which are difficult to detect in blood may be detected in urine. So, more proteins would be identified in urine with the same experimental sensitivity.

Although methods such as multidimensional separation [10] can detect minor proteins or depletion of predominant proteins in plasma, they have several drawbacks. For example, these approaches can introduce additional variability; moreover, because some peptides and proteins bind to certain high-abundance proteins [11], the depletion strategy may lead to a significant loss of information.

While technological limitations prevented the detection of many components of urine in the past, more than 2,300 urinary proteins have been recently identified using high accuracy mass spectrometry [12], and technologies such as CE and SELDI-TOF MS have stimulated research into urinary biomarkers. In addition, urinary proteins containing posttranslation modifications (PTMs) such as glycosylation [13] and phosphorylation [14] are potentially rich sources of disease biomarkers [15]. Moreover, profiling of urine peptides and metabolites also reveals potential for the identification of biomarkers for systemic diseases [16]. Despite this evidence, the importance of urine serving as a biomarker source remains underestimated. For example, we retrieved 316,849 articles in a July 2013 search of PubMed using the keywords [('blood' OR 'serum' OR 'plasma') and 'biomarker'],

compared with 33,930 articles found using [('urine' OR 'urinary') and 'biomarker']. In reality, the number of articles related to protein biomarkers in urine is no more than 450 in a manually organized database (until June 2013).

There have already been some data, which indicates that certain urine biomarkers are of higher quality than those from plasma. For example, urine angiostatin levels have been shown to strongly correlate with the renal pathology chronicity index [17], and urinary levels of ADAM 12 and MMP-9 have been used as noninvasive biomarkers in identifying women at increased risk of developing breast cancer [18]. Moreover, elevation in urinary desmosine levels has been shown to be associated with COPD [19].

Because urine accumulates a significant number of changes, urine samples show a greater degree of variability, particularly in protein concentration and volume [20], even though levels of constitutive proteins remain stable [21]. Such variability can be readily normalized using levels of creatinine [22], cystatin C [23], and N-acetyl-β-D-glucosaminidase (NAG) [24]. A significant challenge for biomarker discovery in urine remains discerning which changes in urine composition were caused by which systematic factor. Questions such as this can be addressed by large-scale population studies to survey normal variations in urine composition, which will lay the foundation for biomarker discovery in urine.

Blood coagulation status is a critical physiological parameter, alterations of which can lead to embolism or hemorrhage. Because the use of blood samples to monitor coagulation status is harmful and invasive, particularly in patients with high risk of spontaneous bleeding, urine samples offer an alternative approach for monitoring the coagulation state that can greatly benefit these patients.

In conclusion, using an identical proteomic analysis approach to profile the protein composition of rat urine and plasma samples before and after treatment with anticoagulants, we found that changes in the abundance of many proteins was consistently detected in urine samples but not in plasma. Urine may be the better source for biomarker discovery, because it accumulates changes, and can be collected noninvasively in large volumes. Faster and better biomarker discovery may lead to more accurate diagnosis and better health care.

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Part II
Urine Collection, Storage and Analysis

Chapter 3
Urimem Facilitates Kidney Disease Biomarker Research

Liu Liu, Lulu Jia and Xuejiao Liu

Abstract Urine is a body fluid that can be noninvasively acquired and contains important biological information about the patient. Urinary proteins are considered to be the best resource of potential biomarkers for kidney disorders. Urinary proteins can be adsorbed to polyvinylidene fluoride (PVDF) or nitrocellulose membranes, which can then be dried and stored in vacuum bag. This membrane is named Urimem. The membrane can even be stored at room temperature for at least weeks without changing the quantity of eluted proteins. With this simple and inexpensive urimem, it is possible to begin preserving urine sample from all consenting patients during each stage of kidney disease development. Thus, the medical research can be conducted more economically, ultimately benefiting the patients who provided the samples. This can potentially change the landscape of medical research and medical practice.

Keywords Urimem · Storage · Urinary proteins

Hard as it is to believe today, a single concept developed by Dr. Henry Plummer at the beginning of the twentieth century changed the face of medicine. The concept

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was a centralized medical record, stored in a single repository, and capable of traveling with the patient [1].

In this century, comprehensive biological sample storage may change the face of medicine again. Biological samples from patients are invaluable for both medical research and medical practice. However, biological samples from patients are not currently preserved as comprehensively or as long as their corresponding medical records, primarily because of the invasiveness, difficulty, expenses, and usefulness that are associated with collecting and storing such samples. Urine is a body fluid that can be noninvasively acquired and contains important biological information about the patient from whom it was obtained. Urinary proteins are considered to be the best resource of potential biomarkers for kidney disorders. There is no other example of a noninvasively accessible body fluid that is so closely associated with a vital organ. Furthermore, because urine is the filtrate of the blood produced by the kidney, urinary proteins can provide not only detailed information about the urinary system but also information about the blood, which reflects the condition of the whole body.

Therefore, urine is an important biological sample that should be preserved for each stage of a disease for each patient. The preservation of a large number of urinary samples for validation is a key step that facilitates biomarker research and the translation from the laboratory to the clinic.

The preservation of urine is commonly performed by freezing and storing it at −80 °C. Due to its large volume and low protein concentration, the storage of urine usually requires a significant amount of space. Urinary proteins may be degraded easier in solution than in complete dry condition.

Simple and inexpensive urinary protein sample preservation can be the starting point for long and comprehensive biological sample storage. Here, we propose a method to absorb urinary proteins on a polyvinylidene fluoride (PVDF) membrane that can then be dried and stored. This method is very simple and inexpensive and requires minimal sample handling. It does not use organic solvents and is environment-friendly. More importantly, the proteins that are bound on the membrane are dry, which prevents their degradation and makes their preservation at room temperature for longer time possible. Because PVDF membranes have a limited protein loading capacity, the most important consideration is that the proteins in one urine sample are all adsorbed into the PVDF membrane to keep the protein pattern faithfully preserved [1].

3.1 Urinary Protein Preservation on the Membrane

1. Determine the urinary protein concentration from a previous urine routine test. Save a portion of the urine for a new urine routine test.
   - — No dilution and 3× dilution;
   - ± 3× and 6× dilution;

+	6× and 30× dilution;
++	30× and 60× dilution;
+++	60× and 120× dilution;
++++	120× and 240× dilution;

Prepare 47-mm-diameter medium-speed qualitative filter paper and PVDF membranes (Immobilon-PSQ Membrane, PVDF, 0.2 μm, 26.5 cm × 3.75 m roll; one PVDF membrane mapping to 4–6 sheets of filter papers).

2. Set the thermostatic centrifuge to 12,000×*g* and the temperature to 4 °C. Centrifuge the diluted urine samples for 10 min and keep the supernatant.
   Optional: Pass 20 ml diluted urine sample through a 0.45-μm filter membrane (Millipore DURAPORE membrane filters, filter type: 0.45 μm HV) with ultra-low protein binding capacity and retain the flow-through.
3. Place 4–6 sheets of wetted circular filter paper on the Vacuum suction filter bottle (10 cm^2 filter area).
4. Place one activated PVDF membrane on the filter paper immediately (before using the PVDF membrane, ensure that it has been activated in methanol and cleaned in pure water), while being careful to avoid the generation of bubbles.
5. Install the vacuum suction filter bottle and fill it with 20 ml supernatant or the flow-through from the 0.45-μm filter membrane.
6. Connect the vacuum suction filter bottle to the vacuum pump and allow the solution to pass through the PVDF membrane dropwise by adjusting the vacuum pressure to approximately 7 kPa. The initial velocity should be approximately 1.3 droplets/second, and the flow rate should decrease until the solution stops dripping. Turn off the vacuum pump. The total filtration time should be approximately 4 min.
7. After being adsorbed to the PVDF membrane, the protein-bound membrane is placed under a bulb with 1,100 W (275 W × 4) of power for 3–4 min to allow drying to completion.
   Optional: The protein-bound membrane is placed in room temperature condition and let it dry to completion naturally.
8. Place the dry membrane with tag paper (recorded information: medical record number, date, and time urine was collected, before or after taking drugs, urine routine test number) into two independent aseptic sealing membranes and store at −80 °C.

Urinary protein elution from the membrane The elution buffer comprised 1 % Triton X-100, 2 % SDS, in 50 mM Tris-HCl, pH 9.5 [2]. Briefly, the protein-bound dry membrane was cut into small pieces and placed in a clean tube to which 0.1 ml elution buffer/cm^2 membrane was added. The membrane in the elution buffer was mixed well by vortexing for 10 min at room temperature and then by ultrasound for 15 min in an ultrasonic cleaner at room temperature. The supernatant was collected by spinning down the membrane. The protein was concentrated using a centrifugal filter with a molecular weight cutoff of 3,000 Da or by protein precipitation with

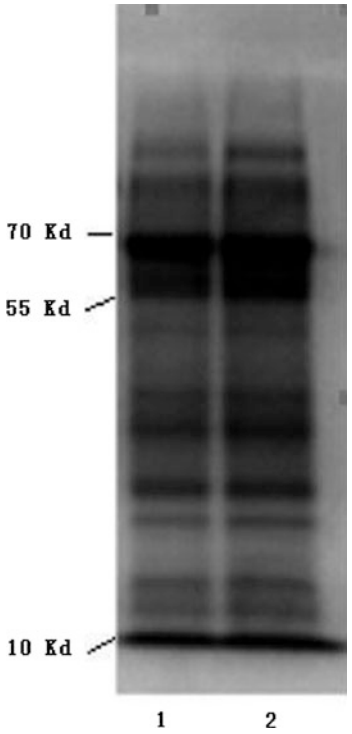

Fig. 3.1 Comparing the urinary proteins after removing cells in urine by centrifugation or filtration, respectively [1]

chloroform/methanol (if the detergent needed to be removed for the downstream analysis such as protein quantification and LC-MS/MS analysis).

Centrifugation or filtration to remove cells in urine As shown in Fig. 3.1, after protein elution from the PVDF membrane with 1 ml elution buffer, 30 μl elution buffer was used for SDS-PAGE. Urine samples that were either passed through a 0.45 μm filter membrane with ultra-low protein binding capacity or centrifuged to remove cells are shown in lanes 1 and 2, respectively. Using a 0.45-μm filter membrane with an ultra-low protein binding capacity conveniently retains cells from the urine sample on the membrane.

Testing the loading capacity of the PVDF membrane As shown in Fig. 3.2, after protein elution from the PVDF membrane with 1 ml elution buffer, 30 μl elution buffer was used for SDS-PAGE. Lanes 1, 3, 5, 7, and 9 represent the eluted proteins from 10, 20, 30, 40, and 50 ml of urine, respectively, in 5 sheets of 10 cm^2 PVDF membrane. Lanes 2, 4, 6, 8, and 10 represent the eluted proteins in the flow-through from the PVDF membrane onto which 10, 20, 30, 40, or 50 ml of urine proteins was absorbed from another 5 sheets of 10 cm^2 PVDF membrane. For urine volumes greater than 30 ml, the protein was not absorbed entirely into the membrane. Thus, the largest urine volume was 20 ml/10 cm^2, and the urine protein concentration was

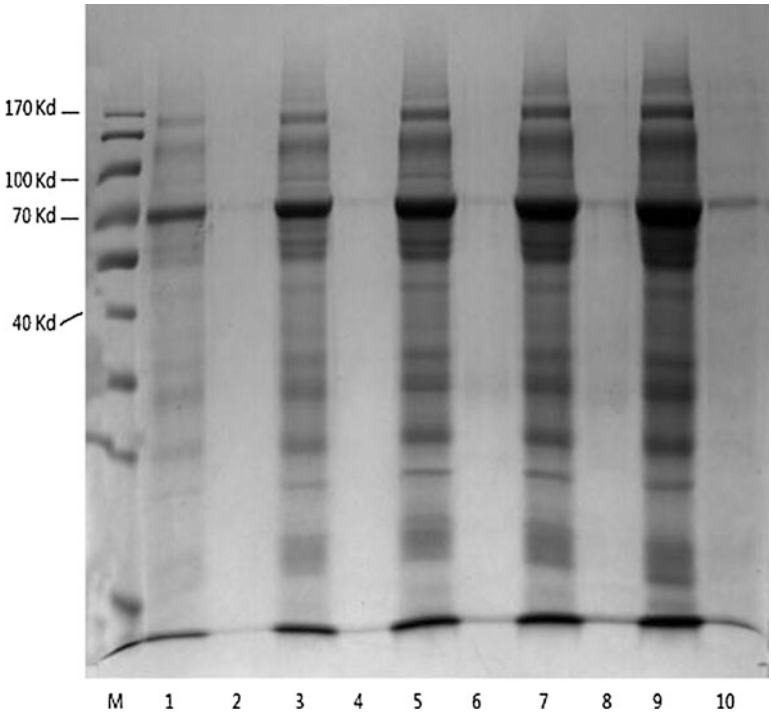

Fig. 3.2 Testing the loading capacity of the PVDF membrane using SDS-PAGE analysis [1]

33 μg/ml. The concentration of the urine protein that passed through the PVDF membrane was less than 66 μg/cm^2.

The urinary proteins recovered from the membrane preserved for 18 days at −80 °C and at room temperature exhibit the same SDS-PAGE pattern Four aliquots of 20 ml of urine proteins were retained on 4 sheets of 10 cm^2 PVDF membranes and stored at four temperature conditions: room temperature, 4, −20, and −80 °C for 18 days. After protein elution from the PVDF membrane with 1 ml elution buffer, 30 μl elution buffer was used for SDS-PAGE. Shown in lanes 1, 2, 3, and 4 of Fig. 3.3 are the proteins that were stored at −80, −20, 4 °C, and room temperature, respectively.

Twenty milliliters of nondiluted proteinuria and 20 ml of 20-fold-diluted proteinuria were filtered through 10 cm^2 PVDF membranes (pore size at 0.22 μm). Then, we precipitated the proteins by chloroform/methanol to remove the detergent. After re-solubilization with lysis buffer (7 M urea, 2 M thiourea, 120 mM DTT, 40 mM Tris-base) and quantification of the protein concentration, equal amounts of protein (25 μg) were separated via SDS-PAGE. As shown in Fig. 3.4, lanes 1 and 2 represent proteinuria and a 20-fold dilution of proteinuria, respectively. The protein patterns were consistent, and no differential bands were observed.

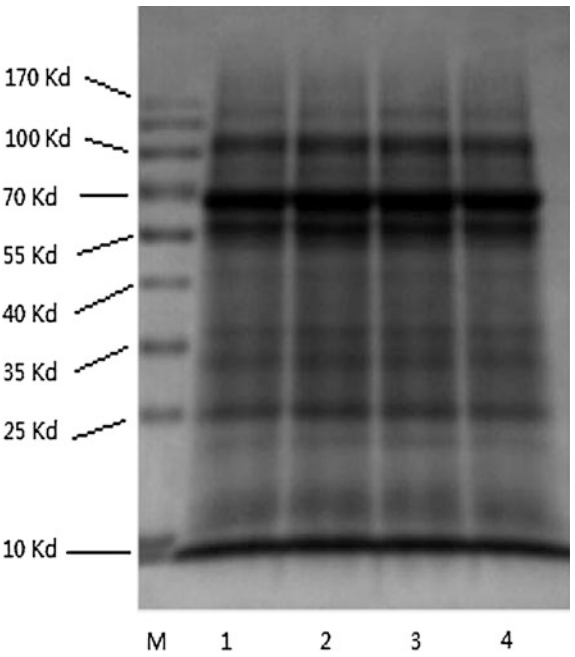

Fig. 3.3 Comparing the urinary proteins recovered from the membranes after preservation for 18 days with different temperature [1]

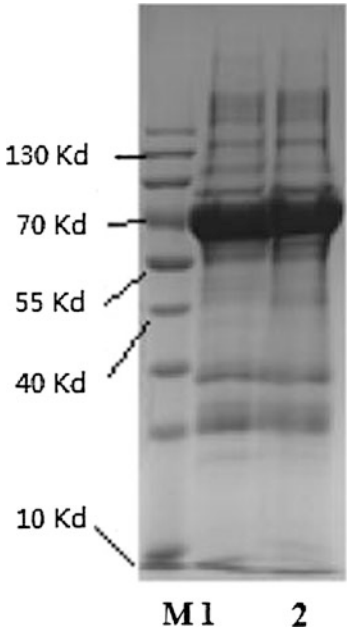

Fig. 3.4 Comparing the urine proteins recovered from the membranes that were loaded onto different amount of urine proteins [1]

This is the first study to report the use of a PVDF membrane to preserve urinary proteins to facilitate biomarker research. Proteins that are preserved on the PVDF membrane are compatible with traditional downstream analytical applications. First, proteins on the membrane can be stained by all commonly used protein stains, such as Ponceau-S Red, Coomassie Brilliant Blue R dye, and Amido Black. The proteins on the membrane can also be visualized by transillumination based on the change of the color of the PVDF membrane from opaque to semi-transparent when wet in 20 % methanol. Based on this result, the total amount of protein on the membrane can be quantified when used in conjunction with densitometry [3]. A second potential application is the immunodetection of the proteins on the membrane by dot blotting. Third, the preserved proteins can be eluted from the membrane for other applications such as western blotting and LC-MS/MS analysis.

By using this simple and inexpensive urinary protein preservation method, it is possible to begin preserving urine sample from all consenting patients during each stage of disease development. However, several considerations must be taken into account when preserving urinary protein samples. A sample taken at a certain time point should be well documented in the patient's medical record. Patient consensus may be required at the time that the sample is taken and also when the sample is analyzed as part of a particular study. As the concept of urinary protein storage is accepted gradually by the medical community, technical standards will likely be developed, and commercial products will likely be produced. It is likely that many new technologies will be developed, including more durable media with improved protein adsorption capabilities; test strips to estimate protein quantity; streamlined protocols for urinary protein collection, drying, sealing, packaging, and labeling; sample storage and management systems for individual sample access and retrieval; and an optimal manner in which to use the membrane-adsorbed protein. Storage at 4 °C or even ambient temperature for longer time periods may be feasible. The use of particular resins might allow small molecules, including creatinine and ions, to be stored economically in the future. Other body fluid such as cerebrospinal fluid can also be stored the same way. In addition, if the cellular structure of blood cells can be compromised, then blood can be blotted without any processing; thus, genetic information will also be available.

Comprehensive historical biological information can also be used in retrospective studies to understand the pathophysiology of disease and the relationships among diseases as well as to monitor the long-term efficacy and side effects of treatments. There will be more ways of extracting and using the information as long as more and more samples are available for research. With this information, medical research can be conducted more easily, considerably faster, and more economically, ultimately benefiting the patients who provided the samples.

We believe, starting from now, it is possible to start preserving urinary protein samples from each stage of disease development for every consenting patient in hospital. This can potentially change the landscape of medical research and medical practice of this century.

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Chapter 4
Human Urine Proteome: A Powerful Source for Clinical Research

Lili Zou and Wei Sun

Abstract As noninvasive and easily available biological fluid, urine is becoming an ideal sample for proteomic study. In recent years, researchers endeavored in profiling urinary proteome and discovering potential disease biomarkers. However, there are still many challenges in the studies of urinary proteome for the complexity of urine. In this article, we review current status of urinary sample preparation, including collection, storage, and extraction of urinary proteins, and the overall urinary proteome analysis so far, which may be helpful for urinary proteome analysis.

Keywords Urinary proteome · Mass spectrometry · Preparation and storage · Normal human

4.1 Introduction

Body fluids have been regarded as the significant source of biomarkers, which could be used for the early diagnosis and state forecast of clinical diseases [1–4]. As an easily obtainable biological fluid, urine plays a crucial role in the clinical proteomics.

Urine is excreted by the kidney to eliminate waste products from plasma. About 150–180 L of plasma is filtered by glomeruli to develop the "primitive urine." More than 99 % of "primitive urine" is reabsorbed by the renal tubule, and the "final urine" is remained to be excreted [5]. Approximately 30 % of urinary proteins originate from the plasma proteins, whereas 70 % comes from the kidney and the urinary tract [6, 7]. Therefore, the urinary proteome might supply important biomarkers directly reflecting the functions of the kidney and related organs [8].

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Urine has several advantages to discover biomarkers of diseases: (1) It is easy to be collected in large amount and noninvasive way and (2) urinary samples are less complex than plasma and carry many proteins, peptides, and amino acids that have not been discovered in plasma [9]. Therefore, many researchers did their best to have a deeper understanding of urinary proteins and discover potential biomarkers in recent years.

However, there still are many difficulties and problems needed to further explore and study. In this review, we summarize achievements of urinary proteomics, including sample collection, preparation, and urinary proteome analysis in recent years, which may be helpful for further studies.

4.2 Collection and Storage

4.2.1 The Types of Urine

In clinic, many kinds of urinary samples have been used according to the different examination. However, many factors may influence the components of urinary proteome [10], including daily activities, physiological variations, and environmental factors such as temperature and pH. Therefore, several types of urinary samples have been analyzed by proteomic approach, including first-morning urine, 24-h urine, second-morning urine, random urine, and the urine collected after drinking a large amount of water or after drinking coffee.

Twenty-four-hour urine can show the excretion of urinary proteins within a day [11]. But the collection of 24-h urine depends on patient compliance, which is unpractical to be completed entirely and easy to have some errors during the collection process [12]. Concerning first-morning urine, it cannot exhibit urine "diurnal variation" (different time-points' variation in all day) [11]. And Hoorn et al. [13] reported that first-morning urine may have bacterial contamination due to the long residence time of bladder.

Sun et al. [10] made a qualitative and quantitative analysis of five samples (first-morning void, second-morning void, excessive water-drinking void, random void, and 24-h void) collected in 1 day from healthy volunteers by 1-D LC/MS. They found no significant differences in the protein numbers of these five samples, and 42 common proteins to five samples contributed an average of 88.7 % of abundance to each sample. Thongboonkerd et al. [14] compared four different time-points urine, including first-morning urine, afternoon urine, water-loading urine, and urine after drinking a cup of coffee. They found the first-morning urine contained greater amount of proteins, but less protein spots visualized in 2D gel than afternoon urine. The water-loading urine had the least amount of proteins by 2D-PAGE analysis, but exhibited a few newly presenting spots. There were more spots in the sample after caffeine ingestion than in water-loading urine.

To avoid bacterial contamination deriving from skin contamination, midstream urine is usually recommended as the standard for urinary proteome analysis, particularly for women [15]. Schaub et al. [16] employed surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in profiling the first-stream urine and midstream urine from three females and three males. For male samples, there were no observable differences between midstream and first-void urines. But for female samples, first-void urine emerged three specific SELDI peptide peaks after 3-day storage compared with midstream urine.

4.2.2 Protease Inhibitor

Protease inhibitors had been initially suggested to be used to prevent proteolysis of clinical biological fluids, which was caused by endogenous proteases [17]. However, whether protease inhibitors are necessary for the studies of urinary proteomics is debatable. Shinada et al. [18] incubated 30-kDa nonglycosylated [^{125}I]IGFBP-3 with urinary samples and found IGFBP-3 proteolysis by SDS-PAGE analysis. But Havanapan et al. [17] studied the effect of protease inhibitors cocktail on midstream random urinary specimens and found that no observable qualitative and quantitative changes by two-dimensional gel electrophoresis (2-DE) analysis. Thongboonkerd [11] suggested protease inhibitors were unnecessary for the studies of nonproteinuric urine because there were lower amount of proteases in urine than in plasma, cells, or tissues.

4.2.3 Preservatives

During the storage, urinary samples might have bacterial overgrowth, which could change the urinary proteome [19]. Therefore, preservatives were recommended to prevent the bacterial overgrowth after collection [19]. Thongboonkerd and Saetun [19] studied the effects of the addition of either sodium azide (NaN_3) or boric acid on bacterial overgrowth in pooled urine from 5 healthy individuals. They found the addition of NaN_3 and boric acid could delay the bacterial overgrowth, and greater delay (for at least 48 h) was obtained by relatively higher preservatives. They recommended addition of 2–20 mM boric acid or 0.1–1 mM NaN_3 to one-void random urinary specimens and addition of 200 mM boric acid or 10 mM NaN_3 to 24-h urinary collections.

4.2.4 Storage Temperature

Appropriate storage temperature could decrease degradation of urinary proteins to some extent. In 1999, Klasen et al. [20] analyzed changes of albumin concentrations after urinary samples were stored at 4, −20, and −70 °C. They found that if samples could be analyzed in 4 weeks after collected, the best storage temperature was 4 °C. And they recommended −70 °C was used as storage temperature for longer storage. They did not suggest storage temperature −20 °C because they found the IgG concentrations decreased after 1-week storage at −20 °C. In 2007, Thongboonkerd and Saetun [19] reported that to prevent the bacterial overgrowth, uncentrifuged urinary samples without preservative should be kept no longer than 20 h at 4 °C. Without any preservative, urinary samples should not be stored at room temperature for longer than 8 h.

4.2.5 Freeze–Thaw Cycle

Previous studies showed freeze–thaw cycles could influence the components of body fluid proteome, including serum/plasma and cerebrospinal fluid [21, 22]. Studies also showed that when urinary samples were stored at low temperature, researchers should avoid freeze–thaw cycles [11]. Schaub et al. [16] analyzed first-void and midstream urinary specimens from three females and three males by SELDI-TOF MS. Results indicated that after 1–4 freeze–thaw cycles, the urinary proteome did not change remarkably except for the loss of intensity in some peaks, whereas some small peaks were undetectable after the fifth freeze–thaw cycle. Powell et al. [11] reported the degradations of some proteins resulted from 4 to 7 freeze–thaw cycles in urinary proteomics.

Furthermore, Klasen et al. [20] found some proteins forming precipitates after storage and thawing. According to the studies of Saetun et al. [23], after overnight storage at −20 °C, urinary proteins may precipitate, and they found that EDTA (5 mM) could reduce the amount of precipitates and pH could influence the type of precipitates. To redissolve the precipitates, effectively shaking of the specimens should be done at room temperature.

4.2.6 pH

Thongboonkerd et al. [24] analyzed adjusted pH urinary samples which were precipitated the proteins by 75 % ethanol. The 2-DE results showed different pH levels did not influence the consistency of individual urinary specimens and the total number of spots. Therefore, they thought it was unnecessary to adjust the pH of urinary samples before gel-based proteome analysis.

4.2.7 Standard Protocol for Urine Collection

Based on previous studies, a standard protocol for urinary collection was recommended by Human Kidney and Urine Proteome Project, HUKPP, and European Urine and Kidney Proteomics, EuroKUP Initiatives from 9 December 2009.

The details were described as follows:

Standard Protocol for Urinary Collection [25]

1. Type of urinary sample
   Midstream of second-morning urine (preferably) or morning random-catch urine, in sterile (preferably) or clean urinary collectors.
2. Pre-treatment and storage
   Centrifuge at 1,000*g*, for 10 min to remove cell debris and casts. Aliquot supernatant avoiding disturbing the pellets at 1.5, 10, or 50 mL (depending on downstream application); Do not overfill the tubes; Store at −80 (preferably) or −20 °C. Record time until freezing (it should be no longer than 3 h).
3. Freezing and thawing
   Avoid freeze–thaw cycles. If thawing and re-freezing occurs, always keep a record of this event.

Notes: http:/www.hukpp.org and www.eurokup.org.

4.3 Urinary Preparation

For urinary proteomics, crude urinary samples are complex including high concentrations of salts, small molecules, and some metabolic wastes [26], and concentration of urinary proteins is too low to be identified. So many sample preparation methods have been applied to concentrate urinary proteins and remove small molecules, such as organic solvent precipitation, ultracentrifugation, dialysis–lyophilization, and ultrafiltration (centrifugal filtration) [26, 27].

4.3.1 Organic Solvent Precipitation

Organic solvent precipitation is a popular method in urinary proteomics. Organic solvent can reduce of permittivity of urine and break hydration shell on the surface of protein molecules, and thus, urinary proteins are gathered and precipitated effectively. Organic solvent precipitation method had several characters. First, this approach can be used to handle larger volumes of urine and takes less time than other methods [27]. Second, it can enrich higher molecular weight proteins species than ultrafiltration [28]. Third, it can effectively precipitate more acidic and hydrophilic proteins than ultracentrifugation [6].

Studies showed types and concentration of organic solvents may play various roles on urinary protein precipitation [14, 27]. Khan et al. [27] employed 2-DE to analyze the urinary proteome precipitated by different organic solvents and found that higher resolution and more protein spots could be obtained using acetonitrile (urine-to-solvent ratio was 1:5). Thongboonkerd et al. [14] made a comprehensive comparison of different organic solvent precipitation methods by 2-DE. They revealed applying 90 or 75 % organic compounds could get greater recovery yield than lower percentage of organic compounds. Ethanol, methanol, or acetone precipitation methods were suggested to obtain more protein spots and higher protein recovery yield in routine or gel-based urinary proteome studies. Moreover, acetonitrile precipitation was suggested for proteinuric urine or a larger volume of urine for its highest number of protein spots but lower protein recovery yield.

4.3.2 Ultracentrifugation

Ultracentrifugation method isolated proteins due to easy sedimentation of high-density protein molecular under ultracentrifugation situation. In 2002, Thongboonkerd et al. [6] analyzed urines from five normal donors by 2-DE and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). They found ultracentrifugation method could fractionate more basic, hydrophobic, and membrane proteins than organic solvent precipitation approach. But ultracentrifugation uses expensive equipment and acidic proteins are lost, which might limit its application [6].

4.3.3 Dialysis

Another commonly used method is to combine dialysis with lyophilization [29]. Oh et al. [29] reported that dialysis–lyophilization approach was likely to profile the whole urinary proteins on 2-DE and could improve reproducibility and resolution, because it could effectively remove the molecules interfered the profiling of 2-DE. Moreover, Thongboonkerd et al. [14] revealed that this method had great protein recovery yield but showed lowest number of protein spots compared with precipitation, ultracentrifugation, and ultrafiltration method.

4.3.4 Ultrafiltration

As for ultrafiltration method, it uses ultrafiltration membranes to discard small molecules and concentrate urinary protein according to molecular weight difference. Court et al. [30] reported that ultrafiltration method enriched lower molecular

weight proteins than 6 % TCA precipitation by SDS-PAGE gel. Based on ultrafiltration method, Vaezzadeh et al. [31] put forward a one-step sample preparation method. They added urinary sample together with Anti-HSA resin to a Vivaspin 6 spin-filter, which could isolate proteins and remove human serum albumin in one step. It realized sample concentration, purification, and albumin depletion simultaneously. Furthermore, they found that neutral pH (7–8) could achieve both of efficient depletion and high protein recovery.

4.4 Normal Human Urinary Proteomes Analysis

Many researchers had undertaken studies to catalog the normal human urinary proteome. The first study came from Anderson et al. [32] in 1979. They found 250 urinary protein spots by 2-DE. But without high-throughput protein identification approach, it was hard to profile the components of urinary proteome. The development of two ionization methods in MS, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), made the precise analysis of biomacromolecule possible [33]. In 2001, Spahr et al. [28] firstly employed LC-MS approach to analyze the human urinary proteome and identified 124 urinary proteins. After that, many groups contributed their efforts to profile a comprehensive normal human urinary proteome. 2-DE and LC-MS were two popular approaches for proteome analysis.

4.4.1 2-DE Approach

In 2002, Thongboonkerd et al. [6] reported their study utilizing acetone precipitation and ultracentrifugation preparation methods. By 2-DE and MALDI-TOF, they identified 47 unique proteins, 28 from acetone precipitation method and 19 from ultracentrifugation method.

In 2004, Oh et al. [29] prepared urinary samples by dialysis–lyophilization and removed albumin using Affi-Gel Blue. They identified 113 urinary proteins on 2-DE by peptide mass fingerprinting with MALDI-TOF-MS analysis. In the same year, Pieper et al. [7] reported a large-scale urinary proteome analysis. First, they fractionated urinary proteins by size exclusion chromatography and collected two fractions, higher than 30-KDa and lower than 30-KDa. Then, they employed immunoaffinity subtraction chromatography to remove albumin and immunoglobulin G from higher than 30-KDa fractions. At last, the two fractions were separated by 2-DE. A total of 1,400 distinct protein spots were found, and 420 spots of these were identified to 150 unique protein.

In 2005, Smith et al. [34] collected 35 urinary samples from 12 donators and extracted the urinary proteins by solid-phase extraction method. By 2-DE and MALDI-TOF/TOF analysis, 48 nonredundant proteins were identified.

In 2006, Khan et al. [27] used ultrafiltration and different organic solvent precipitation method to isolate urinary proteins, and a total of 339 proteins were found with 2-DE separations followed by MALDI-TOF analysis. Zerefos et al. [35] exploited preparative electrophoresis to separated urinary proteins, and by 2-DE and MALDI analysis, 778 protein spots were found and 141 proteins were identified.

4.4.2 LC-MS

In 2002, Pang et al. [36] applied 2DLC-MS method and identified 51 urinary proteins from normal human urinary proteome.

In 2005, Sun et al. [37] applied three approaches to analyze the urinary proteome, 1DE plus 1DLC-MS, direct 1D LC-MS and 2DLC-MS. They identified 226 urinary proteins, 171 proteins of which were identified for the first time. Castagna et al. [38] used hexameric peptide libraries methods to reduce the high-abundant proteins and enrich medium and low abundant ones in urinary proteome. By this method, they identified 383 unique proteins and 251 proteins were not ever found.

In 2008, Lee et al. [39] handled urinary samples by four different approaches: vacuum centrifugation, 90 % ethanol precipitation, microconcentrator, and reverse-phase trapping column. By in-gel digestion and LC-MS analysis, 154, 154, 162 and 148 proteins were identified, respectively, in four preparation methods and 600 proteins were found in total [40].

In 2009, Kim et al. [41] modified isoelectric focusing and asymmetrical flow field-flow fractionation by applying Teflon tubing to connect multilane asymmetrical flow field-flow fractionation (AF4) channel with isoelectric focusing (IEF) channel (prevent the possible protein adsorption by membrane wall of IEF segments). The fractions from IEF were analyzed by LC-MS and 245 urinary proteins were identified.

Urinary protein posttranslational modification was an important issue for urinary proteome analysis. In 2006, Wang et al. [42] utilized concanavalin A to enrich N-linked glycoproteins from normal urinary proteome. By 1DE plus 1DLC-MS and 2DLC-MS, a total of 225 glycoproteins were identified, 150 annotated as glycoproteins by Swiss-Prot and 43 by NetNGlyc 1.0.

4.4.3 High-Resolution MS Analysis

Along with great improvement of mass accuracy of mass spectrometer, new generations of high-resolution MS dramatically increased protein identification for proteomics [43].

In 2006, Jun Adachi et al. [44] reported the first urinary proteome analysis by high-resolution MS. They analyzed in-gel and in-solution digestion urinary samples by LTQ-FTICR and LTQ-Orbitrap. By combining 1,281 proteins from LTQ-FTICR

with 1,055 proteins from LTQ-Orbitrap, a total of 1,543 urinary proteins were obtained from this in-depth study. Gene Ontology (GO) analysis exhibited that membrane proteins occupied nearly half of the annotated proteins. Extracellular proteins were overrepresented and intracellular proteins were underrepresented. However, plasma membrane proteins and lysosome proteins were unexpectedly overrepresented.

In 2010, Goo et al. [45] analyzed the urinary samples from ten female healthy persons by LC coupled with a hybrid linear ion trap–orbitrap mass spectrometer and identified 1,003 urinary proteins. Li et al. [46] used urines from three healthy male donors, and digested peptides were fractionated by two approaches, integrated multidimensional liquid chromatography and Yin-Yang multidimensional liquid chromatography methods. 6,739 unique peptides and 1,310 nonredundant proteins were obtained by two approaches. Furthermore, they did the first large-scale work to profile urinary phosphoproteome and found 45 unique phosphopeptides from 31 phosphoproteins. Most of the phosphorylation sites were on serine residues except for six on threonine and only one on tyrosine residues.

In 2011, Marimuthu et al. [40] reported the first urinary proteome result of high-resolution MS/MS with LTQ-Orbitrap Velos mass spectrometer. They exploited in-gel digestion and LC-MS approach to analyze unfractionated proteins of the pooled urine, as well the glycoproteins after the lectin affinity enrichment. 1,452 proteins were found in unfractionated urine and 617 proteins in glycoproteome. A total of 1,823 proteins were found, and 671 proteins of these proteins were identified in human urine for the first time. Two hundred and sixty-five proteins out of 617 enriched proteins were glycosylated. Forty-four peptides out of 131 peptides identified with protein N-terminus were analyzed to be acetylated.

4.5 Conclusion and Outlook

Following the development of MS technologies, precision proteomics become more and more significant in proteomics. It could not only reveal more proteins secreted in urine but also avoid more errors which lead to misdirected results [50]. Especially, in 2011, Marimuthu et al. [47] published the first urinary proteome study with both of MS and MS/MS at high resolution exhibiting more credible results. With the application of urinary proteome to clinical researches, a larger precision urinary proteome database should be developed, which should be used as a reference for further study.

Another important issue for urinary proteome was high-throughput quantitation. Quantitation of urinary proteins has been proposed, and many approaches were exploited to realize relative and absolute quantitation. In 2013, Nolen et al. [48] applied multiplexed bead-based immunoassays and made absolute quantitation of 211 proteins in healthy urinary samples. However, more than 600 proteins could be identified in only one 1DLC-MS run [49]. Therefore, high-throughput urinary protein quantitation, especially absolute quantitation, still needs more concern.

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Chapter 5
Exosomes in Urine Biomarker Discovery

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Abstract Nanovesicles present in urine the so-called urinary exosomes have been found to be secreted by every epithelial cell type lining the urinary tract system in human. Urinary exosomes are an appealing source for biomarker discovery as they contain molecular constituents of their cell of origin, including proteins and genetic materials, and they can be isolated in a non-invasive manner. Following the discovery of urinary exosomes in 2004, many studies have been performed using urinary exosomes as a starting material to identify biomarkers in various renal, urogenital, and systemic diseases. Here, we describe the discovery of urinary exosomes and address the issues on the collection, isolation, and normalization of urinary exosomes as well as delineate the systems biology approach to biomarker discovery using urinary exosomes.

Keywords Exosome · Urine · Biomarker · Systems biology

5.1 Introduction

Extracellular nanovesicles called "exosomes" are small (20–100 nm) membrane vesicles that, in mammals and invertebrates, are secreted by a wide variety of cell types [1]. Exosomes are formed inside their secreting cells in endosomal compartments called multivesicular bodies (MVBs). Exosomes are released from the MVB

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lumen into the extracellular environment upon fusion of the outer membrane of the MVBs with the plasma membrane. Exosomes contain various molecular constituents of their cell of origin, including proteins and genetic materials, i.e., mRNA and miRNA. The potential roles of exosomes in intercellular communications have been studied in immunology, neurobiology, stem cell, and tumor biology. In 2004, exosomes in human urine were identified and partially characterized [2]. Urinary exosomes contain proteins that are characteristic of every epithelial cell type facing the urinary space, including multiple protein products of genes responsible for renal and systemic diseases. Urinary exosomes provide a non-invasive means of acquiring unique information about the physiological or pathophysiological state of the renal cells of their origin; many studies have been performed aimed at identifying urinary biomarkers of specific diseases [3–12]. In this chapter, we discuss the discovery of urinary exosomes; collection, isolation, and normalization of urinary exosomes; and the potential of urinary exosome constituents in biomarker discovery.

5.2 Discovery of Urinary Exosomes

Over 15 years ago, integral membrane-bound proteins, such as water channel aquaporin-2 (AQP2), could be detected in low-density membrane vesicles isolated from human urine by ultracentrifugation [13, 14]. The presence of these proteins in urine allowed studies of water-balance disorders [15]. However, the mechanism by which these AQP2-containing vesicles actually got into urine was unknown. Further investigations determined that other membrane-bound transporters were detectable in urine low-density membrane fractions [16], including major Na transporters of the proximal tubule (the type 3 Na–H exchanger [NHE3]), the thick ascending limb of Henle's loop (the bumetanide-sensitive Na–K-2Cl cotransporter [NKCC2]), and the distal convoluted tubule (the thiazide-sensitive Na–Cl cotransporter [NCC]). Based upon these initial observations, researchers sought to discover the mechanisms behind the release of these so-called low-density membrane vesicles into the urine.

In 2004, a pivotal study used a variety of techniques to characterize the orientation, size, and protein contents of low-density urinary membrane vesicles isolated from normal human subjects by differential centrifugation [2]. These studies allowed the authors to hypothesize that AQP2 and other apical membrane-bound transporter proteins were being excreted into the urinary space through the process of exosome formation, i.e., a process where the internal vesicles of multivesicular bodies (MVBs) are delivered to the urinary space by fusion of the outer membrane of MVBs with the apical plasma membrane of renal tubular epithelial cells. To demonstrate the orientation of proteins within the low-density urinary vesicles, the authors used immunogold electron microscopy and antibodies against epitopes on the cytoplasmic side of the integral membrane proteins AQP2 and NCC, or antibodies against epitopes on the external side of aminopeptidase N and CD9. Analogous to exosomes found in other bodily fluids, characteristic of vesicles found

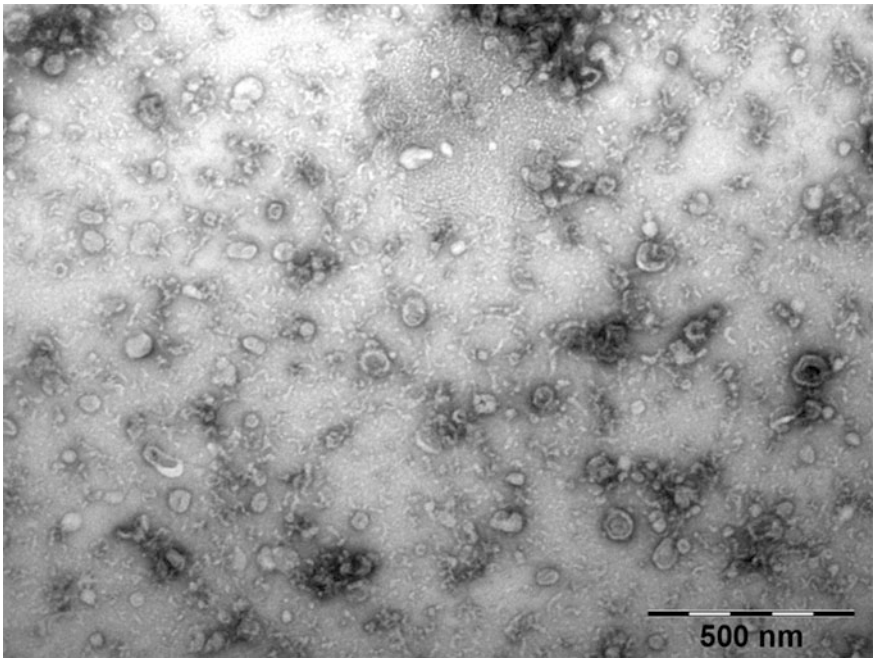

Fig. 5.1 Negative staining of urinary exosomes from normal human subjects using electron microscopy at 41,000× magnification. This magnification was used to show a variety of exosomes from a 200,000×*g* low-density pellet based on the 20–100 nm size and round, cup-like shape

in MVBs, and the membrane proteins within low-density urinary vesicles were oriented with the cytoplasmic-side inward. Negative stained electron micrographs revealed that the low-density urinary vesicles were small, cup-shaped nanovesicles between 20 and 100 nm in diameter (Fig. 5.1), and a quantitative analysis showed that the mode of the vesicle size was 35–40 nm [2]. These findings were consistent with the size criterion of exosomes proposed by Thery and colleagues and similar to that of exosomes found from other tissue types in the human body [17]. Finally, Pisitkun et al. [2] analyzed the proteome of the low-density urinary vesicles using nanospray liquid chromatography–tandem mass spectrometry (LC-MS/MS). Over 290 unique proteins were identified with a role in a wide range of cellular processes and included proteins from renal epithelia extending from the glomerular podocytes through the proximal tubule, the thick ascending limb of Henle's loop, the distal convoluted tubule, and the collecting duct as well as from the transitional epithelium of the urinary bladder. Twenty-one of the identified proteins are associated with kidney diseases or hypertension. Importantly, the proteins identified included 73 endosomal trafficking proteins and many class E vacuolar protein-sorting (VPS) proteins. These VPS proteins including members of the endosomal sorting complexes required for transport (ESCRT) are well-established proteins associated with MVB biogenesis and exosome formation (Fig. 5.2) [18].

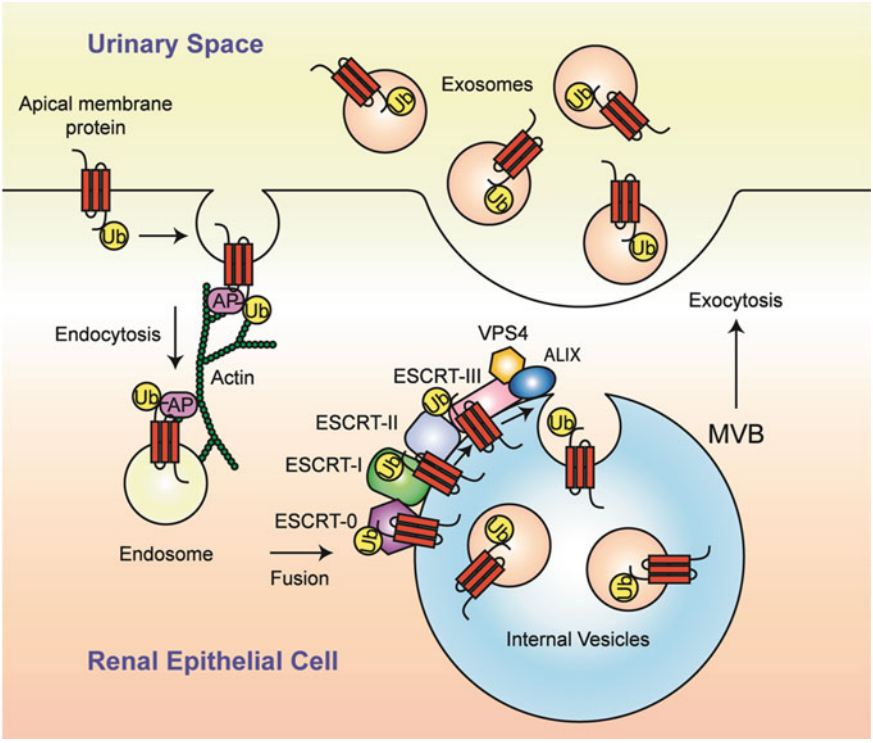

Fig. 5.2 A model for multivesicular body (MVB) formation and exosome secretion into urine. Monoubiquitination (Ub) is the signal that marks plasma-membrane proteins for incorporation into MVBs. Monoubiquitinated proteins are endocytosed by a process dependent on adaptor proteins (AP) and sorted in the endosomal pathway. Fusion with and internalization of the ubiquitinated cargo into the MVB requires assistance from endosomal sorting complexes required for transport (ESCRT)-protein machinery (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III), VPS4, and ALIX. Eventually the outer membrane of the MVB fuses with the apical plasma membrane of the renal epithelial cell, releasing exosomes into the urinary space

Based on these data, the authors concluded that low-density urinary vesicles are largely made up of exosomes derived from the internal vesicles of MVBs of every epithelial cell type facing the urinary space (Fig. 5.2). Importantly, urinary exosomes contain proteins that potentially reflect the physiological or pathophysiological state of their cells of origin, and therefore, exosome isolation, as highlighted by the authors, may provide an efficient first step in biomarker discovery in urine.

To expand their initial findings, in 2009, the same investigators conducted a large-scale proteomic study of human urinary exosomes using a more sensitive LC-MS/MS system. Overall, 1,132 proteins were identified including 14 phosphoproteins. The Online Mendelian Inheritance in Man (OMIM) database highlighted that 177 of these proteins were disease-related proteins, 34 of which were associated with renal diseases and/or hypertension [19]. An online database of human urinary exosomal proteins can be found at http://dir.nhlbi.nih.gov/papers/lkem/exosome/.

These studies showed proof-of-principle that the proteomic analysis of urinary exosomes can serve as an excellent tool for discovering disease-related biomarkers.

In addition to a wide range of proteins [2, 19, 20], Miranda et al. [5] discovered in 2010 that nucleic acids, including mRNA and microRNA (miRNA), were contained and preserved in urinary exosomes. The identified mRNA species encoded proteins from all segments of the renal tubule. These studies demonstrated that exosomal genetic materials have potential roles for urine biomarker discovery and that exosomal RNAs may have functional roles, i.e., in cell-to-cell communication along the nephron [21].

5.3 Collection, Isolation, and Normalization of Urinary Exosomes

5.3.1 Collection and Storage of Urine Samples

For clinical and research purposes, it is important that urine destined for isolation of exosomes is collected and stored in a standardized way. There are three important steps that should be addressed to standardize the collection and storage of urine: (1) addition of protease inhibitors and preservative during urine collection, (2) storage of urine at stable temperatures, for short-term and long-term use, and (3) extensively vortexing urine samples after thawing [12, 22] (see more at http://www.niddk.nih.gov/research-funding/at-niddk/labs-branches/kidney-disease-branch/renal-diagnostics-therapeutics-unit/sample-collection-storage-exosome-analysis/Pages/default.aspx). Without the use of protease inhibitors, various key proteins often detected in urinary exosomes can degrade. The typical cocktail of protease inhibitors and preservative includes phenylmethanesulfonyl fluoride (PMSF), leupeptin, and sodium azide. From our own experiences, storage of urine at −80 °C provides a more stable condition than storing samples at −20 °C. With extensive vortexing after thawing, samples stored at −80 °C have the highest recovery of exosomes—up to 100 % when compared to freshly processed urine. In contrast, urine stored at −20 °C has only 87 % exosome recovery after extensive vortexing. Nanoparticle tracking analysis of urinary exosomes has recently confirmed a storage condition of −80 °C with the addition of protease inhibitors is the best [22]. Urine collected in the morning, both first and second urine of the day, has similar exosome contents and can be used interchangeably for experimental research purposes [12].

5.3.2 Processing Procedures for Isolation of Urinary Exosomes

After the discovery of potential renal disease-related biomarkers in urinary exosomes, researchers have worked to identify a variety of methods for faster and more

efficient ways to collect urinary exosomes for clinical applications. This exploration began because the first method of isolation, ultracentrifugation, requires long processing times and access to expensive equipment. Despite the applications of novel techniques, including membrane filtration and exosome precipitation methods, ultracentrifugation methods show the highest exosome purity. Table 5.1 shows a comparison of urinary exosome isolation techniques.

Ultracentrifugation was the original method of reproducibly isolating urinary exosomes [2]. This technique requires collecting urine in the collection solution

Table 5.1 Comparison of urinary exosome isolation techniques

	Methods	Advantages	Disadvantages	References
Ultracentrifugation	Ultracentrifugation	• Reproducible results • High yield of intact proteins and nucleic acids	• 4 to 5 h to process single sample • Some contamination of highly abundant proteins • Expensive equipment	[2]
	Double-cushion ultracentrifugation	• Less contamination of highly abundant proteins • Reproducible results	• Long processing time • Tedious separation techniques • Expensive equipment	[23]
	Sucrose gradient ultracentrifugation			[24]
	Ultracentrifugation—size exclusion chromatography			[25]
Membrane Filtration	Nanomembrane filtration	• Shorter processing time, 0.5–2 h • Many samples can be processed at one time • Relatively inexpensive • Can be used in a clinical setting	• Possible clogging of membrane • Sample loss • Contamination of highly abundant proteins	[3]
	Micromembrane filtration			[26]
Precipitation	Precipitation by ExoQuick-TC	• Shorter processing time, 0.5–2 h • Yields intact RNA • Relatively inexpensive • Can be used in a clinical setting	• Low purity of protein • Modified protocol	[27]

(protease inhibitors and sodium azide), spinning the urine at a low speed (17,000×*g*) to remove whole cells, large membrane fragments, and cellular debris, followed by spinning the supernatant at a high speed (200,000×*g*) for 1 h to pellet the exosomes (for more details please check http://www.niddk.nih.gov/research-funding/at-niddk/labs-branches/kidney-disease-branch/renal-diagnostics-therapeutics-unit/exosome-preparation/Pages/default.aspx). This initial low-density exosome pellet commonly includes some contamination of highly abundant urinary proteins, including albumin and uromodulin, also known as Tamm-Horsfall protein (THP) [2, 23]. THP forms double-helical fibrils by a disulfide cross-link zona pellucida (ZP) domain, which entraps exosomes and is co-isolated with the low-density pellet. Adding the reducing agent dithiothreitol (DTT) with a subsequent high-speed spin reduces the presence of THP in the low-density pellet [2]. However, this method does not completely decontaminate the sample of all THP and other abundant proteins. Alternatively, a more purified exosome pellet can be obtained using an additional step of double-cushion ultracentrifugation [23] or the use of heavy water and a sucrose gradient in ultracentrifugation [24] to separate the exosomes and contaminating proteins based on density, whereas ultracentrifugation followed by size exclusion chromatography (UC-SEC) can be used to separate contaminating proteins from exosomes based on molecular weight [25]. However, these methods are still time-consuming and labor intensive and require expensive equipment.

In 2007, Cheruvanky et al. [3] used commercially available nanomembrane concentrators to filter exosomes from urine of healthy volunteers and proteinuric patients with focal segmental glomerulosclerosis (FSGS). The exosome isolation time was reduced from 4 h using standard ultracentrifugation to 0.5–2 h using nanomembrane filters [3]. Using this system, the authors were able to detect various proteins typically found in urinary exosomes, even from patients who had high quantities of contaminating proteins, demonstrating that this method could be used for both clinical and routine experimental applications. Other microfiltration methods have also been shown to provide an efficient way of isolating urinary exosomes with reduced contamination of highly abundant urinary proteins as compared to nanomembrane filtration and ultracentrifugation techniques [26]. Alternatively, precipitation methods have been used to isolate urinary exosomes. In 2012, Alvarez et al. [27] showed that a commercially available exosome precipitation kit called ExoQuick-TC could be used to isolate urinary exosomes, although a modified protocol provided improved results. This modified exosome precipitation method was shown to provide a more efficient yield of miRNA and mRNA than protein compared to sucrose cushion ultracentrifugation.

5.3.3 Normalization

A challenge that researchers in the urinary exosome field face is defining the methods of exosome normalization. Without standardized normalization protocols, biomarker discovery studies from urinary exosomes and the subsequent comparisons

between patient-to-patient samples will be less reliable. There are several methods of normalization, including time normalization, creatinine normalization, and protein normalization.

The quantitative measurement of urinary biomarkers in terms of excretion rate is the optimal method for normalization and has been used for many years for analysis of classical urinary biomarkers such as total protein and albumin (e.g., expressed in g/day or μg/min). Assessments of urinary biomarkers based only on concentrations are unsatisfactory because normal physiological variations in water excretion can dilute or concentrate urinary proteins making this measurement unreliable for both intra- and interpersonal comparisons. Time normalization, the collection of urine from patients within the same bracket of time, is thus the most accurate method of measuring urine exosomal protein excretion rates and is the most reliable method of comparing patient exosome products side-by-side. An even more accurate time normalization would be the collection of all urine from the same patient over 24 h, but this approach has several practical limitations.

As there are difficulties acquiring time-normalized urine samples, and this method also rules out using urine samples from a biobank because these samples are typically spot urine samples, a more common method of normalization, creatinine normalization, is used in a clinical setting. Creatinine is typically excreted in urine at a steady rate. The average 24-h urine creatinine excretion rate for the general population is approximately 1,000 mg/day per 1.73 m^2 body surface area. Given the amount of creatinine in the spot urine, an estimated rate of exosome excretion can be assigned. However, this method has limitations; it does not take into account actual difference in individual creatinine excretion rates or renal diseases that make creatinine excretion rates unstable, such as acute kidney disease.

Normalization based on the amount of a particular protein in urine has been proposed, including the uses of THP, exosomal markers (e.g., ALIX or TSG101), or other biomarker proteins that when measured together provide a concentration ratio that correlates with disease state [6, 28]. However, further studies are needed to confirm the validity of these normalization methods. An alternative method of normalization that has recently been utilized is counting exosome number. Using a new nanoparticle tracking analysis system, urinary exosomes in whole urine were counted, sized, and analyzed [22]. This new method of analysis may ultimately be less time-consuming and provide a more standardized normalization procedure, yet comes with the disadvantage of requiring expensive equipment.

5.4 Urinary Exosomes in Biomarker Discovery

Urinary exosomes provide a non-invasive method of discovering novel biomarkers for renal diseases. A technique that has become popular over the past decade for the discovery of novel biomarkers in the renal system and exploration of the urinary exosomal proteome is LC-MS/MS [7]. A technical description of LC-MS/MS is beyond the scope of this chapter (see review by Pisitkun et al. [29]), but a brief

description is informative for the context of this section. LC-MS/MS is highly sensitive and can be used for both qualitative and quantitative analysis of peptides/proteins. Qualitative approaches involve observing the absence or presence of a particular protein in a given sample, while quantitative approaches show a comparison of the relative abundance of specific proteins. LC-MS/MS-based quantification of patient and control samples can be performed side-by-side using label-free [30], label-based (e.g., iTRAQ [31] and TMT [32]), and the more recent targeted quantification [33]. To validate and compare proteins of interest discovered by LC-MS/MS from urinary exosomes, Western blots, ELISA, or immuno-electron microscopy are commonly used.

The paradigm of utilizing a systems biology approach for clinical and translational research in the field of urinary exosome biomarker discovery is gaining momentum. The systems biology approach using mass spectrometry generally includes three phases: (1) discovery, (2) validation, and (3) implementation (Fig. 5.3) [7]. In the discovery phase, LC-MS/MS is used to identify unknown biomarkers and assist in developing hypotheses for the following phases. Typically, a limited number of well-defined patient samples are used to identify quantitative or qualitative differences in protein or mRNA/miRNA expression. Animal models are often exploited to show physiological changes within a specific experimental condition, e.g., increasing the excretion of AQP2 in urine from rats with elevated vasopressin levels by administering dDAVP or thirsting [14]. Once candidate biomarkers have been identified, the validation phase can begin.

A variety of promising biomarkers from urinary exosomes have been identified from many renal, urogenital, and systemic diseases. Some biomarkers of acute kidney injury (AKI), urogenital cancer, chronic kidney diseases, glomerular diseases, renal allograft rejection, and unique tubulopathies are summarized in Table 5.2. In addition to being a great source of protein biomarkers, exosomes also contain functional mRNA and miRNA, which markedly expands the potential repertoire of biomarkers. In recent studies, for example, mRNA and miRNA in urinary exosomes were used to identify potential markers for prostate cancer and renal fibrosis [34–36]. In many cases, early detection of a disease or injury is required for increasing the chances of successful treatment, and several of the biomarkers already discovered could provide early detection possibilities. For example, patients in the intensive care unit or patients undergoing heart surgery have a high risk of acute kidney injury that may increase morbidity and mortality.

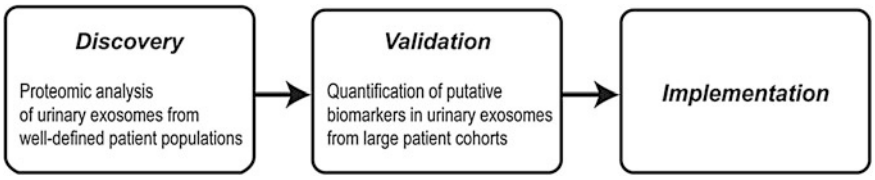

Fig. 5.3 Systems biology workflow paradigm showing the three major steps required for the development of urinary biomarker assays for routine clinical application

Table 5.2 Potential biomarkers for renal and systemic diseases from urinary exosomes

Groups	Diseases	Urinary exosomal proteins	Urinary exosomal RNAs	References
Acute kidney injury	Acute kidney injury (AKI)[a]	Fetuin-A		[11]
	Acute kidney injury (AKI)	Activating transcription factor 3		[10]
	Renal ischemia-reperfusion injury	Aquaporin-1		[37]
Cancer	Prostate cancer		PCA-3 and TMPRSS2: ERG	[36]
	Bladder cancer[a]	CD36, CD44, trophoblast glycoprotein, basigin, and CD73		[40]
	Non-small cell lung cancer (NSCLC)[a]	Leucine-rich α-2-glycoprotein		[41]
	Renal cell carcinoma[a]	Matrix metalloproteinase 9, ceruloplasmin, podocalyxin, Dickkopf-related protein 4, carbonic anhydrase IX, aquaporin-1, extracellular matrix metalloproteinase inducer, neprilysin, dipeptidase 1, and syntenin-1		[42]
	Bladder cancer[a]	Tumor-associated calcium signal transducer 2		[43]
Chronic kidney disease	Renal fibrosis		microRNA-29c	[34]
	Renal fibrosis		CD2AP	[35]
	Chronic kidney disease	Osteoprotegerin		[44]
Glomerular disease	Puromycin-treated rats or podocin-Vpr transgenic mice and from patients with focal segmental glomerulosclerosis	Wilms tumor 1		[10]
	IgA nephropathy versus thin basement membrane nephropathy[a]	Aminopeptidase N, vasorin precursor, α-1-antitrypsin, and ceruloplasmin		[45]

(continued)

Table 5.2 (continued)

Groups	Diseases	Urinary exosomal proteins	Urinary exosomal RNAs	References
	Diabetic nephropathy[a]	Xaa-Pro dipeptidase, major urinary protein 1 and neprilysin		[46]
	Diabetic nephropathy	Wilms tumor 1		[47]
	Podocyte injury in FSGS or SSNS	Wilms tumor 1		[48]
Misc.	Liver injury[a]	CD26, CD81, SLC3A1, and CD10		[49]
	Light chain amyloidosis (Al)	Immunoglobulin light chain species		[50]
Renal Transplantation	Renal allograft recipients with acute decrease in renal function[a]	Several pairs of biomarkers		[6]
Tubulopathy	Aldosteronism	Phosphorylated NCC and prostasin		[9]
	Pseudohypoaldosteronism type II (PHAII)	Total and phosphorylated NCC		[38]
	Gitelman syndrome	Total and phosphorylated NCC		[38]
	Gitelman syndrome	NCC		[39]
	Bartter syndrome type I[a]	NKCC2		[19]
	Post-obstructed kidney	Aquaporin-1 and transforming growth factor β1		[51]
	Salt sensitivity of blood pressure		45 exosomal microRNAs	[52]

[a] indicates studies that used LC-MS/MS for discovery

Proteins in urinary exosomes such as Fetuin-A [11], activating transcription factor 3 (ATF3) [10], and aquaporin-1 [37] have been shown to change significantly in the early phase of patients with AKI compared to controls and could be promising markers for early detection of AKI.

Biomarkers for genetic diseases, such as Gitelman syndrome [38, 39] and Bartter syndrome type 1 [19], which have mutations in the SLC12A3 gene (encoding NCC) or the SLC12A1 gene (encoding NKCC2), respectively, have been investigated in urinary exosomes. Little to non-detectable levels of these proteins were observed in exosomes from these patients. Early detection of the diseases could guide appropriate treatments and prognosis. This innovation may replace cumbersome functional tubular tests, i.e., 24-h urinary electrolyte excretion. Furthermore, the absence in detection of NCC or NKCC2 protein in urinary exosomes could

directly reflect the abnormalities in gene products and be more physiological relevant than genetic testing methods.

Despite many potential biomarkers having been discovered, these identifications are only the first phase in the systems biology paradigm—discovery. In the validation phase, potential biomarkers are quantified in large patient cohorts, with the sensitivity and specificity of each candidate biomarker assay evaluated. It is important to note that the assays developed in the validation phase should be used in the implementation step, which requires the development of easy, time-efficient, and affordable assays to use in a clinical protocol. Currently, there are a limited number of translational studies investigating the validity of biomarkers from urine. One example is a recent study investigating pseudohypoaldosteronism type II (PHAII) and Gitelman syndrome, where an ELISA was developed as a practical clinical tool to detect total NCC and phosphorylated NCC in urinary exosomes of patients with PHAII and Gitelman syndrome as well as a large patient pool with a variety of clinical backgrounds [38]. This study revealed that the ELISA yielded similar sensitivity as immunoblotting technique, thus providing a practical diagnostic tool for detecting changes in urinary NCC excretion. Although this study has potential, including the generation of a sensitive immunoassay, the protocol still requires laborious ultracentrifugation for the isolation of urinary exosomes. An increased commitment to validate promising biomarkers from urinary exosomes and develop a user-friendly clinical assay is needed in order to bring diagnostic tools from the bench to finally "implement" at the bedside.

5.5 Summary and Recommendations

Characteristics of exosomes Urinary exosomes have similar characteristics of exosomes secreted from other cell types in the body, including exosomes released by dendritic cells and B-lymphocytes. These defining characteristics include that proteins in the membranes of urinary exosomes have an orientation of cytoplasmic-side inward; urinary exosomes are small in size, between 20 and 100 nm in diameter; and urinary exosomes contain several class E vacuolar protein-sorting (VPS) proteins known to be involved in MVB biogenesis and exosome formation, along with many endosomal trafficking and membrane proteins. Urinary exosomes contain numerous proteins associated with renal and systemic diseases and thus are a great resource for biomarker discovery. Nucleic acids also contribute to the biological makeup of urinary exosomes and should be considered during biomarker discovery.

Collection and storage Urine, following the addition of protease inhibitors, can be stably stored at −80 °C over long periods of time. Following extensive vortexing, this storage condition provides an exosome recovery rate up to 100 %. First and second morning urine contains similar exosome content and can be used interchangeably for research purposes.

Isolation of exosomes There are a variety of methods available for isolating human urinary exosomes. Although ultracentrifugation is laborious, it is a standard method that yields intact exosomes, with reproducible results, that can be used for many research techniques. To decrease contamination of abundant urinary proteins such as albumin or Tamm-Horsfall protein, additional steps of ultracentrifugation with heavy water and a sucrose gradient or double-cushion ultracentrifugation can be used to separate proteins based on density. Ultracentrifugation followed by size exclusion chromatography can be used to separate contaminates based on molecular weight. However, for large-scale, clinical protocols that call for isolation of urinary exosomes, membrane filtration, and precipitation methods may be a better choice because many samples can be processed in a shorter amount of time. These quicker methods could increase the discovery rate of disease-related biomarkers.

Normalization To accurately compare side-by-side patient or control exosome samples, 24-h urine collection or timed urine collection yields the most accurate results based on the excretion rate of exosomal biomarkers. However, when this type of controlled collection is not available, creatinine and protein normalization can be used. The new technology of nanoparticle tracking could play an important role in developing a new normalization protocol.

Biomarker discovery and clinical implementation Urinary exosomes provide a non-invasive method of identifying potential protein and mRNA/miRNA biomarkers for renal and systemic diseases. LC-MS/MS is a common method used to discover urinary biomarkers because it is highly sensitive and can be used for both quantitative and qualitative analysis. A systems biology approach to biomarker discovery includes three phases: (1) discovery, (2) validation, and (3) implementation. Many potential biomarkers for early detection of different renal disorders, genetic disorders, and systemic diseases have been discovered. However, many of these biomarkers have not made it to the translational stage of validation or implementation. There is a need in the field to validate these biomarkers for clinical and routine use.

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Chapter 6
Urinary Proteins with Post-translational Modifications

Liu Liu and Xuejiao Liu

Abstract Research on the human urine proteome may lay the foundation for the discovery of relevant disease biomarkers. Posttranslational modifications (PTMs) have important effects on the functions of protein biomarkers. Identifying PTMs without enrichment adds no extra steps to conventional identification procedures for urine proteomics. The only difference is that this method requires software that can conduct unrestrictive identifications of PTMs. These PTMs include methylation, dehydration, oxidation, hydroxylation, phosphorylation, or dihydroxylation. These data are useful reference for PTM biomarker discovery in the future.

Keywords Posttranslational modification · Urine proteome · Unenriched

Research on urine proteomics is important for the discovery of disease biomarkers. Posttranslational modifications (PTMs) of proteins regulate many physiological functions. For example, acetylation is an important PTM in metabolism regulation; phosphorylation is an important PTM in regulating enzyme activity in cellular signaling pathways; oxidation is an important marker of cellular aging; and methylation is an important PTM in the regulation of gene expression. PTMs of proteins are subject to change, and these proteins may be potential disease biomarkers. As reported previously, in patients with diabetes, there are many advanced glycation end product peptides in urine [1, 2]. The urine glycoproteomic makeup is altered in

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patients with chronic kidney diseases [3]. It has been shown that changes in osteopontin PTMs in urine are related to kidney stones and ovarian cancer [4, 5]. Further, 2D gels have demonstrated that there are different molecular masses of the same protein in the urine proteome [6]. Mass spectrometric immunoassays of urine protein phenotypes have also revealed a novel glycated end product of β-2-microglobulin [7].

Previous studies of urine protein PTMs have focused primarily on glycosylation, in which the proteins were first enriched via glycosylation and then identified as glycosylated proteins [8–10]. With enrichment, PTMs can be detected with high sensitivity. Research on other types of PTMs has been limited by the lack of enrichment methods [11] because each method can only identify one type of PTM. In the present study, instead of enriching for any specific PTMs, conventional urine proteomics techniques were used, and unspecified PTMs of urine proteins were identified with the MODa and PEAKS 6 software. Without enrichment, sensitivity to identify the PTMs is low. Thus far, only one previous study on urine proteomics reported the identification of phosphorylated proteins without enrichment [12].

In conjunction with recent developments in PTM research, dozens of expert algorithms have been created to perform unrestrictive searches of protein PTMs that can find almost all known PTMs and even novel PTMs. In this study, the PTM algorithms in the software packages MODa and PEAKS were used. MODa enables fast "multi-blind" unrestrictive PTM searches with a speed that is an order of magnitude faster than other existing approaches. It can also identify any number of modifications on a single peptide. In contrast to alternative approaches, MODa simultaneously uses multiple sequence tags from each MS/MS spectrum and a dynamic programming algorithm to identify modifications between sequence tags matched to a database peptide [13]. PEAKS PTM is an improved software tool for peptide identification with unspecified PTMs. The improvements in this software include a default setting whereby the software considers all PTMs included in the universal protein resource (Unimod) database as variable PTMs. Moreover, several search strategies are employed to reduce the search time [14]. PEAKS PTM was included in the PEAKS 6 software, which is the only commercial software that can identify unspecified variable PTMs.

In the study reported last year [15], real in vivo PTMs were isolated from other PTMs including in vitro PTMs and acid substitutions by a manual search; the in vitro PTMs are mostly created during experimental processes. In all, 39,144 spectra with 6,194 unique peptides and 1,994 proteins were identified by MODa. Among these, 7,100 spectra with 1,602 unique peptides and 734 proteins contained PTMs with sizes accepted by the MODa search regardless of the modification classification in Unimod. Within these PTMs, 433 spectra with 169 unique peptides and 85 proteins had in vivo PTMs. Furthermore, 47,857 spectra with 9,878 unique peptides and 1,606 protein groups were identified by PEAKS 6. Among these, 20,329 spectra with 3,891 unique peptides and 1,578 proteins had PTMs with sizes accepted by the PEAKS 6 search regardless of the modification classification in Unimod. Within these PTMs, 880 spectra with 254 unique peptides and 182 protein groups had in vivo PTMs. These findings are summarized in Table 6.1.

In this search, 15 types of in vivo PTMs were identified by MODa and 10 types of in vivo PTMs were identified by PEAKS 6 (Table 6.2).

Table 6.1 A summary of the spectra, unique peptides, and protein numbers [7]

	Software	#PSMs(peptide spectrum match)	#Peptides	#Proteins
Whole urine	MODa	39,144	6,194	1,994
	PEAKS 6	47,857	9,878	1,606[a]
PTMs	MODa	7,100	1,602	734
	PEAKS 6	20,329	3,891	1,578
In vivo PTMs	MODa	433	169	85
	PEAKS 6	880	254	182
Percentage of in vivo PTMs in whole urine (%)	MODa	1.106	2.728	4.263
	PEAKS 6	1.839	2.571	11.333

[a] In PEAKS 6, a protein represents a group of proteins sharing all identified peptides

Table 6.2 A summary of the names, modification sizes, and modification sites of all the in vivo PTMs, as well as the number of spectra, unique peptides, and proteins with in vivo PTMs [7]

In vivo PTMs	Software	ΔMass	Position	#PSMs	#Peptides	#Proteins
Oxidation or hydroxylation	MODa	16	CDKNPRY	204	105	40
	PEAKS 6	15.99	DKNPRY	224	139	71
Methylation	MODa	14	CDEHKNSQRT	106	58	27
	PEAKS 6	14.02	DEILNT, C-term, N-term	157	160	99
Dehydration	MODa	−18	ST	26	19	14
	PEAKS 6	−18.01	STY	102	81	67
Dihydroxy	MODa	32	CLMPT	29	1	10
	PEAKS 6	31.99	KPRY	32	48	30
Phosphorylation	MODa	80	DS	20	8	2
	PEAKS 6	79.97	ST	192	154	57
Acetylation	MODa	42	STM(Protein N-term)	7	5	5
	PEAKS 6	42.01	CST, Protein N-term	39	41	46
Hydroxymethyl	MODa	30	N	3	1	1
Pyrophosphorylation		160	S	16	2	1
Lysine oxidation to aminoadipic semialdehyde		−1	K	12	6	6
Deamidation		1	R	7	4	4
Didehydro		−2	SY	6	1	1
HexNAc	PEAKS 6	203.08	NST	141	19	43
Carboxylation		43.99	E	10	6	7
Persulfide		31.97	D	7	6	8
Hexose		162.05	T, N-term	4	14	14

Table 6.3 The in vivo PTMs identified by both software packages and the number of peptides and proteins [7]

In vivo PTMs	#Peptides	#Proteins
Oxidation or hydroxylation	34	10
Methylation	22	11
Dehydration	10	5
Dihydroxy	2	1
Phosphorylation	5	2
Acetylation	2	1
All	75	25

There are significant number of PTMs identified by both MODa and PEAKS 6 (Table 6.3). The peptides with in vivo PTMs identified by both MODa and PEAKS 6 were screened out because the proteins identified as containing these peptides were somewhat different between the two software packages. Detailed data can be found in the paper [15].

This is the first study of its kind to identify PTMs in the urine proteome without preferential enrichment, using a mixture of 12 human urine samples (6 males and 6 females). The pooled sample was used to identify as many PTMs as possible in a single experiment. Because the original donors that provided the urine samples may differ in gender, age and other medical conditions, the PTMs in the urine proteomes are also likely to be different among the individuals. The PTMs in individual urine samples will be studied in the future. Moreover, the reagents from the experimental procedures including protein digestion may introduce many artifact PTMs that are not endogenous to the samples. For example, urea can cause the non-enzymatic modification of carbamylation to certain proteins. The two software packages identified both artifact PTMs and in vivo PTMs. We manually excluded all possible artifact PTMs and reported only the unequivocal in vivo PTMs.

These data may provide a useful reference for biomarker discovery in the future. As the technology and algorithms for conducting proteomic screens improve, more PTMs from the urine proteome will likely be identified.

6.1 Methods

Urine collection, protein digestion, and LC/MS/MS analysis were done as previously reported without special need. Data were processed as following, and it can be modified and improved as the software development.

6.1.1 Software and Operating Environment

MODa was obtained from the Division of Computer Science and Engineering of Hanyang University in Korea by email eunokpaek@hanyang.ac.kr. A trial version of PEAKS 6 was downloaded from the Bioinformatics Solutions Web site. The

operating environment for MODa was a computer with 2 GB RAM and an Intel® Core™2 Duo CPU E6750@2.66 GHz 2.00 GHz. PEAKS 6 was operated on a computer with 16 GB RAM and an Intel® Xeon® CPU X5650@2.67 GHz 2.66 GHz (2 processors).

6.1.2 File Conversion

The RAW files were converted to MGF files by the MM File Conversion software.

6.1.3 Database

The Uniprot human proteomics database released on March 21, 2012.

6.1.4 Parameters for the MODa Search

According to the README instruction in the software folder, the parameters were set as follows:

PeptTolerance = 2.5: This parameter indicates the parent mass tolerance in Daltons.
AutoPMCorrection = [0|1]: The default parameter value is "0," whereas "1" means that the program will automatically find the optimal parent mass for the input spectrum, regardless of the specified PeptTolerance.
FragTolerance = 0.5: This parameter indicates the fragment ion mass tolerance in Daltons.
BlindMode = 2: This parameter indicates the number of modifications per peptide, and "2" allows an arbitrary number of modifications per peptide.
MinModSize = [−200], maxModSize = [+200]: This parameter indicates the minimum and maximum modification size in Daltons (Da).
Enzyme = Trypsin, KR/C: This parameter indicates the reagent used for protein digestion as well as the cleavage sites and amino acid terminus.
MissedCleavage = [2]: This parameter indicates the number of allowed missed cleavage sites.
CysteineBlocking = Carbamidomethyl, 57: This parameter indicates the chemical derived from a free cysteine by the alkylation process and the mass of the chemical derivative.
False discovery rate (FDR) ≤1 %: This parameter indicates the FDR of the peptide–spectrum matches (PSMs).

6.1.5 Parameters for the PEAKS 6 Search

The search parameters were set as follows:

Parent mass error tolerance: 10.0 ppm
Fragment mass error tolerance: 0.1 Da
Precursor mass search type: mono-isotopic
Max missed cleavages: 2
Non-specific cleavage: 1
Fixed modifications: carbamidomethylation: 57.02
Variable modifications: deamidation (NQ): 0.98; oxidation (M): 15.99; Pyro-glu from Q: −17.03; 4-hydroxynonenal (HNE): 156.12; acetylation(K): 42.01; acetylation(N-term): 42.01; acetylation(protein N-term): 42.01; amidation: −0.98; and 669 more built-in modifications in PEAKS 6
Max variable PTM per peptide: 3.
Result filtration parameters: de novo score (ALC%) threshold: 30; peptide −10 lgP ≥ 17.5; protein −10 lgP ≥ 20; FDR (peptide–spectrum matches): 1.00 %.

6.1.6 Manual Search

For MODa, the observed modification size was matched with the modification name and classification on the Unimod Web site (http://www.unimod.org/modifications_list.php). The modification size was set as the average mass. The modification size tolerance was set as 0.05 Daltons. For PEAKS 6, the observed modification name was matched with the modification classification on the Unimod Web site. Some of the PTM classifications in Unimod are artifact, posttranslational, chemical derivative, AA substitution, pretranslational, and multiple. The PTMs that are classified as "Posttranslational" represent in vivo PTMs.

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Chapter 7
Applications of Peptide Retention Time in Proteomic Data Analysis

Chen Shao

Abstract In proteomic studies, liquid chromatography is commonly used to separate peptide mixtures prior to mass spectrometry (MS) detection. As an independent dimension of information from the information provided by the MS, peptide retention time information has been proven to be able to aid proteomic data analysis in many aspects. So far, some popular software has offered options for this information for MS data acquisition and analysis. This chapter is a brief review of current methodologies of retention time prediction and application in proteomic analysis.

Keywords Retention time · Peptide identification · Quality control

7.1 Retention Time Prediction

A peptide's retention time (RT) is defined as the length of time elapsed from the injection of a sample into the chromatography system to the detection of peak maximum of a peptide. It depends on its chemical structures of peptides, along with the interaction between the environment (mobile and stationary phase, temperature, pH, etc.). Therefore, peptide RTs in a particular liquid chromatography (LC) condition can be predicted based on chemical structure-related properties of peptides, such as amino acid composition, sequence, hydrophobicity, and other physicochemical properties [1].

The task of RT prediction is to calculate a retention scale for each peptide in the given LC condition, e.g., to calculate the hydrophobicity scale in reverse-phase LC. A simple idea is to measure or predict retention coefficients for individual amino

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acids, and then, the retention scale of a peptide is predicted as the sum of retention coefficients of its constituent amino acids. The amino acid retention coefficients can be predicted either by a set of synthetic peptides with residues substituted by each of the twenty amino acids [9] or linear regression models based on peptides with various amino acid compositions [2, 21, 22, 31].

In the recent years, prediction models were refined by employing peptide sequence information and more intelligent computational algorithms, as well as large size of datasets that could prevent the problem of overfitting in data training [16, 27]. N-terminal residues were found to be influence factors to peptides' retention behavior due to the ion-pairing retention mechanism [19]. Taking into account of this effect, Krokhin et al. developed a widely used prediction model, sequence-specific retention calculator (SSRCalc) [16]. This model added a series of sequence-related correction factors to the previous model that predict peptide retention scales by the summation of individual amino acid retention coefficients [9]. Besides three of the N-terminal residues, these correction factors included C-terminal residues, nearest-neighbor effect of charged side chains (Lys, Arg, and His), peptide length, isoelectric point, hydrophobicity, propensity to form helical structures, etc. Another comprehensive model was built by Petritis et al. [27] based on artificial neural network. Similar to SSRCalc, their model embodied peptide properties such as length, sequence, nearest-neighbor amino acids, hydrophobicity, and hydrophobic moment, as well as predicted secondary structures as the input nodes of the neural network. Some other prediction models were developed in similar idea, but with different choices of peptide properties and statistical models [15, 29, 23].

The refined modes improved the prediction accuracy (R^2) significantly from approximately 0.91–0.92 to 0.96–0.98 [17]. However, these conclusions were based on limited size of datasets and reported by the authors themselves. A blind comparison of the most updated versions of prediction models would help greatly in the selection of proper prediction model for practical use. Besides, considering that models based on sequence information and intelligent computational algorithms often require a lot of computational time and large size of training datasets, the simpler and linear prediction models that provide less, but also sufficient prediction accuracy may be selected in some cases, such as on-the-fly RT prediction and calibration [10].

7.2 Application of RT Information in Proteomic Analysis

7.2.1 Peptide Identification Based on LC-MS Data

Accurate mass and time tag (AMT tag) is a well-known strategy to identify peptide sequences based on LC-MS data, which was firstly invented to identify the *Deinococcus radiodurans* proteome [34, 38]. Given the fact that many possible peptide species are unlikely to be detected in a particular biological system, this strategy assumes that peptides that are detectable in a biological system can be separated by a two-dimensional mass and RT vector [44]. Two main steps are included in this

strategy. In the first step, an AMT database for a particular organism or type of biological sample is constructed based on high-confident peptide identifications from previous replicate LC-MS/MS analysis. Secondly, peptides are identified from LC-MS experiments by matching measured mass and normalized elution time (NET) features to the existing database.

There are similar methods that are also identify peptides based on the accurate measurements of mass and RT [11, 24, 41]. These methods do not need to construct a reference database prior to peptide identification. Instead, features are matched by measured mass and RT between different LC-MS/MS runs. Then, peptide identifications from MS/MS spectra can be transferred from one single run to the others. In a study of urinary proteome [25], using "match between runs" option implemented in MaxQuant software [3], the authors were able to increase number of protein identifications from an average of 462 to 633 in a single run.

Saving the effort from MS/MS analysis, AMT tag and similar methods can improve the efficiency and coverage of proteomic analysis. The success of these methods depends on the complexity of biological system as well as the resolution of both MS instruments and LC systems. False discovery rate (FDR) or confidence of peptide identification can be estimated by decoy database searching (shifting masses of all peptides in the AMT database by a certain value) [28] or statistical models [20, 37, 43]. Study of computational simulation showed that for organisms with relative small proteomes, such as *Deinococcus radiodurans*, modest mass and RT accuracies were sufficient for confident peptide identifications by the AMT tag strategy. For more complex proteome, such as human proteome, more strict criteria should be used. The majority of proteins could be uniquely identified within the tolerances of 1 ppm for mass and 0.01 for NET [26].

7.2.2 Peptide Identification from MS/MS Spectra

RT information has been used to improve peptide identification from MS/MS spectra in several ways. One strategy is to incorporate RT information into a discriminant function along with other peptide-spectrum matching parameters, such as SEQUEST scores [39]. This discriminate function was trained based on data from a known protein mixture. When applying to human plasma proteome analysis, it achieved a 16 % increase of positive peptide identifications.

Predicted RT information can serve as a validation parameter for peptide identification results generated by database searching programs. Kawakami et al. [12] validated peptide identifications by the correlation between measured and predicted RTs. Peptide identifications within a certain correlation tolerance were accepted as high-confident identifications. Several studies reported that number of true positive peptides increased significantly by the combination use of RT filter and lower threshold of database searching score [15, 29, 33].

Besides the application of predicted RT information, Sun et al. built up an empirical RT database based on high-confident peptide identifications from

repeated LC-MS/MS runs of a urine sample [40]. This database was used to validate MS/MS identifications for new urine samples. The bottleneck of the empirical database method is that it can only be applied to peptides that were previously detected in a particular proteome, whereas every peptide sequence can have a predicted RT value. However, this method still has its value because it avoids the problem of incorrect RT prediction, which is evitable due to the complex nature of peptide retention behavior.

7.2.3 Post-translational Modification Identification

PTM on a peptide alters not only its molecular mass, but also its physicochemical property (e.g., hydrophobicity), resulting in RT shifts. The RT difference between modified and unmodified peptide (ΔRT) provides a new dimension of information in additional to mass shift (ΔM) in PTM identification.

Previous studies reported lots of instances that peptides with different modification types or different modification sites elute in different RTs [4, 13, 32, 42]. Zybailov et al. [45] depicted the ΔRT distributions of dozens of modification forms detected in a plant proteome. They found that the direction of RT shifts correlated well with the hydrophobicity shifts of the modified peptides for the majority of modifications. Combination of ΔRT and ΔM constrains can efficiently reduce the FDR in PTM identification [32], especially for studies on low-resolution mass spectrometers. For example, deamidation of a peptide results in a mass shift of only 0.984 Da, which could not be accurately distinguished from its unmodified form by a low-resolution LCQ mass analyzer. A study [4] based on synthetic peptide pairs observed that deamidated peptides elute about 3 min later than the corresponding unmodified forms in RPLC. Deamidation detection accuracy was improved from 42 to over 93 % by filtering original SEQUEST identifications by both ΔRT and ΔM constrains.

ΔRT information was also used to improve the algorithms for fast search of unrestricted modifications. The Delta Accurate Mass and Time (DeltAMT) algorithm [7] calculates a two-dimensional delta vector (ΔM, ΔRT) for each pair of spectra obtained in a LC-MS/MS run. The whole set of spectrum pairs are composed of two classes, those from modified and unmodified forms of the same peptide and those from two unrelated peptides. Thus, there are two classes of delta vectors, modification-induced ones and random-induced ones. Bivariate Gaussian mixture models are employed to discriminate modification-induced distributions from random ones. Then, putative modifications could be identified and reported with (ΔM, ΔRT) information as well as the putative modified and unmodified spectrum pairs. Since this algorithm does not use any fragment ion information from MS/MS spectra, it is able to find out high-confident modifications in a very fast speed. However, this algorithm is limited to high abundant modifications, since vector distributions of low abundant modifications are not usually distinguishable from random ones.

7.2.4 Time-scheduled Targeted Proteomic Analysis

Multiple reaction monitoring (MRM) is the method of choice in targeted proteomics. It is a highly sensitive method for accurate quantitation of low abundance proteins in complex protein mixtures. This method needs a sufficient dwell time for each transition to maintain sensitivity and a reasonable cycle time to ensure accurate quantitation. Thus, only a limited number of transitions can be measured in each cycle, limiting its throughput [30]. Time-scheduled transition acquisition (tMRM) offers a solution that can remarkably increase the throughput of traditional MRM experiment without compromising its performance. In this method, the whole gradient time is split into small time windows, and transitions are monitored only in selected windows centered around the expected RT of peptides. Thus, with the same dwell time setting and number of transitions monitored in each duty cycle, tMRM is able to measure many times of transitions in the whole gradient time [36].

A key point to the success of tMRM is to define proper RT window that can capture the entire peptide elution profile from baseline to baseline. This depends on accurate prediction of peptide RTs for each injection. In spite of strict control of the LC system, RT shifts between injections are inevitable, especially when experiments lasting for days to weeks to analysis large amounts of samples. To fit in with the RT shifts, predefined RT windows need to be regularly corrected or repredicted, reducing the efficiency and robustness of tMRM experiment. To aid this situation, on-the-fly RT calibration methods have been developed and integrated in the instrument operating software [8, 14].

This method makes use of a set of well-characterized landmark peptides to calibrate RTs of targeted peptides. Landmark peptides could be either spiked-in synthetic peptides [6, 8] or endogenous peptides that distribute in a broad range of the whole gradient. At any time point, RT windows of subsequent targeted peptides are adjusted based on a local linear regression model generated by the last two eluted landmark peptides. RT windows of peptides elute between the first and second landmark peptides can be simply adjusted by RT shift of the first landmark peptide to calibrate the difference in dead volume. Broad RT windows are set for all landmark peptides as well as peptides elute before the third landmark peptide to ensure that they can be captured without or with minimal calibration.

This method achieved over 90 % success rates on analyses of 180 targeted peptides in a gradient from 0.5 to 2 % solvent B per minute, as well as a nonlinear gradient [8]. It could also precisely correct RT shifts caused by other factors such as change of loading amounts of samples [6] and different LC columns [14]. This method significantly increases the robustness of the entire tMRM workflow by compensating for several commonly occurred changes in experimental conditions, reducing the requirement of LC reproducibility in analysis. Researchers can be rescued from offline RT calibration of LC system and refinement of RT prediction models, saving experimental time, and importantly, precious biological samples.

7.3 Discussion and Perspective

It has been well proven that using RT information could benefit proteomic data analysis. However, its application in practical proteomic analysis has so far been restricted because RT information is of lower resolution compared to mass information, and importantly, peptide RT alters in different LC conditions. Krokhin and colleagues addressed this issue by optimizing their SSRCalc prediction model by four popularly used LC conditions in proteomics. These LC conditions are 300 Å-TFA, 100 Å-TFA, 100 Å-formic acid, and 100 Å-pH 10 [5, 16, 18]. However, since there are hundreds of choices of mobile and stationary phases and other LC parameters in practice, it is an impossible task to pretention retention scales for all LC conditions. A more flexible solution is to train and test the prediction model in the same LC run [15]. Theoretically, this solution is able to adapt all LC conditions. The limitation of this solution is that it needs a sufficient set of high-confident peptide identifications for model training, which is not always available in a single LC run. Another prediction model, ELUDE, is the combination of the above two solutions [23]. When sufficient data are available, ELUDE derives a new RT index for the condition at hand; otherwise, it selects and calibrates a pretrained model from a library of predictors. Model selection and calibration processes are performed automatically by robust statistical methods in ELUDE, facilitating its practical use. However, it should be noted that the accuracy and efficiency of all prediction models are still needed to be tested blindly by datasets covering a great variety of LC conditions.

LC alignment is another important technology in this field. Slight changes of LC conditions and inevitable RT shifts between LC runs can be adjusted by this technology [8]. A recent review of LC alignment methods can be found at [35]. A good idea is to employ a set of spiked-in synthetic peptides as landmarks for LC alignment or to correlate predicted retention scales and measured RTs for each run. These peptides are designed to span a wide range of hydrophobicity, allowing accurate alignment for the entire LC profile. For example, six synthetic peptides were employed to optimize the SSRCalc model in different LC conditions (2009); the eleven iRT standard peptides were used for on-the-fly RT calibration in tMRM analysis [6].

To use RT information as a parameter in data analysis, a proper tolerance value or window size should be set up firstly. This depends on the experimental reproducibility heavily. The wider the RT window is, the more false positives would be achieved. Therefore, there is also an urgent need to set up standards and quality control methods for LC experiments. With the joint effort of bioinformaticists and experimental biologists, RT information would be widely used in practical proteomic analysis in the near future.

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Chapter 8
Urine Sample Preparation in 96-well Filter Plates to Characterize Inflammatory and Infectious Diseases of the Urinary Tract

Yanbao Yu and Rembert Pieper

Abstract Urine has been an important body fluid source for diagnostic and prognostic biomarkers of diseases for a long time. Technological advances during the last two decades have enabled a fundamental shift from the discovery of candidate protein biomarkers using single-assay platforms to highly parallel liquid chromatography tandem mass spectrometry (LC-MS/MS)-based proteomic analysis platforms. MS/MS-based approaches such as multiple reaction monitoring (MRM) are also being used increasingly for targeted protein biomarker validation. In large part due to the fact that the majority of protein in voided urine is soluble, such studies have focused on the analysis of urine supernatants, whereas the pellets were discarded after centrifugal sedimentation. Urine sediments are of particular value in the analysis of urinary tract infections (UTI). The LC-MS/MS methods now have sufficient resolving power and sensitivity to survey metaproteomes—the entirety of proteins derived from multiple organisms that interact with each other in mutualistic or antagonistic fashion. Challenges of proteomic analysis of urine include the high dynamic range of protein abundance, high levels of protein post-translational modifications, and high quantities of natural protease inhibitors. Recently, a robust and scalable workflow that can parallelize the processing of multiple urinary supernatant and sediment samples was developed and validated in our lab. This method utilizes 96-well format filter-aided sample preparation (96FASP) strategy and was shown to successfully identify large numbers of proteins from urine samples. Processing 10–50 μg total protein in single experiment, LC-MS/MS with a Q-Exactive mass spectrometer resulted in more than 1,100 distinct human protein identifications from urine supernatants, and around 400 microbial and 1,400 human protein identifications from urine sediments. The surveys are a rich data resource not only for biomarker discovery but also to interrogate mechanisms of pathogenesis in the urinary system.

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8.1 Introduction

Urine is a biological fluid amenable to routine clinical evaluation. Urine has a unique advantage over other body fluids such as blood plasma, cerebrospinal fluid, and bronchoalveolar lavage fluid in that it is accessible non-invasively and in large quantities [3, 6]. As a result of blood plasma filtration in the glomeruli of the kidneys, many proteins circulating in the blood are also present in urine. This fact provides an opportunity to survey biomarkers for pathological conditions of not just the urinary system but essentially all organs of the body [2, 5, 7, 10, 21]. Urinary proteomic studies reported to date usually focus on the analysis of proteins in the urine supernatant following a typically low speed centrifugation step to compact and discard the urinary sediment [1, 10]. The urine supernatant contains soluble proteins and at least a fraction of the urinary exosomes released from uroepithelial and tubular epithelial cells [13]. Urinary sediments have been examined microscopically and in urine culture for several decades to diagnose disease of the urinary system, particularly urinary tract infections (UTI) [15]. Proteomic studies of urinary sediments have been performed only recently in the context of UTI and exploratory assessment of a urinary microbiome [4, 20]. Urinary sediments (the term pellet is used here synonymously) are a source of evidence of hematuria (e.g., red blood cells in the urine), leukocyte infiltration of the urinary tract, and renal and uroepithelial cells shed as whole cells or casts into the urinary tract lumen. These processes are more prevalent in the presence of inflammation and infection. Other small molecules such as crystallized organic and inorganic salts, sugars, and ketones may also be preset in urine sediments. Urinary sediments may contain microbial cells if the urinary tract is colonized by microbial species. Bacterial colonization is far more common than fungal or parasitic colonization. In UTIs, the sediments also contain human host proteins associated with immune response against the invading pathogen(s). The presence of proteins participating in the immune response can be surveyed via proteomics and can serve to distinguish infection from asymptomatic bacteriuria [4]. The immune responses during UTIs have been previously characterized extensively and reviewed [12, 18, 20]. Almost 170 proteins identified in the urinary pellet of a UTI patient (14 % of all identified proteins) were involved in inflammatory responses and wound healing [20]. Bacteria considered commensal organisms in the vaginal tract not inducing inflammatory responses in the urinary tract were also identified from urinary pellets [4]. Analysis of the human host proteome and microbial organisms in urinary pellets promise to provide higher level of insights into pathogenic mechanisms, microbial co-existence, and host–pathogen interactions in the urinary tract. If the methods can be simplified and performed more cost-effectively, they may serve directly as a diagnostic method for UTIs.

Different approaches have been taken to analyze urinary proteomes, many of which have shown promising results. However, urinary proteomic analysis still remains challenging because of inherent protein diversity, dynamic range, and lack of homeostasis, the latter of which results in high inter- and intra-individual variability of the urinary proteome [11]. For instance, the protein abundance in urine was reported to span over five orders of magnitude, and just top-17 most abundant proteins account for 50 % of the total protein mass [20]. The overwhelming signals of those highly abundant proteins may dominate the LCMS analysis and then reduce the possibilities of detecting low abundant proteins in urine. Adachi et al. [1] used SDS-PAGE-based fractionation approach and a hybrid linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer to identify more than 1,500 unique proteins from urine supernatants. Kentsis et al. [6] and Marimuthu et al. [9] performed extensive fractionations of urine supernatants using gel, ion exchange, reverse phase, and lectin enrichment based approaches and identified 1,823 and 2,362 proteins, respectively, with high confidence in protein identifications. Metaproteomic analysis of urinary pellets searching MS data with protein sequence databases corresponding to multiple genomes allowed identification of hundreds of proteins from microbial and human origin [4]. Using a Q-Exactive system, the same methodology resulted over 2,200 proteins identifications from urinary pellets of UTI patients [20]. Notably, in this analysis, no fractionation or depletion of abundant proteins was employed prior to LC-MS/MS analysis running 130 min standard reverse phase nano-LC gradient.

Some of the publications cited in the previous paragraphs used or adapted a filter-aided sample preparation (FASP) method [8, 19] to profile urinary proteomes. In this method, an ultrafiltration/concentration device (usually with a 10 kDa or 30 kDa MWCO) is filled with a protein solution or lysate and then followed by buffer exchanges to remove chemicals or reagents interfering with subsequent reduction, alkylation, and enzymatic digestion steps. The resulting detergent-free proteins are not recovered as usual; instead, direct on-filter digestion is performed. After digestion, protein fragments (peptides) typically have mass less than 5 kDa. Therefore, they pass through the filter membrane upon centrifugation. Because FASP allows flexible use of detergents and buffers to lysis cells and could generate LCMS-friendly samples, this approach has widely been used for basic researches and biomarker discoveries. In clinical proteomic projects, it is desirable to process a large number of samples in order to achieve the statistical power necessary for identifying potential biomarkers. Most FASP-based applications reported to date process samples individually. Development of a 96-well based FASP method was first reported in 2013 [17]. This method was re-examined and advanced further with respect to proteome depth and repeatability using urine samples and named 96FASP [20]. Batch-mode operation of up to 96 samples is achievable and reduces the requirement of experimental repetition and the overall costs. Here, a detailed 96FASP protocol to process urine samples for LC-MS/MS analysis is presented, including procedures for urinary supernatants and sediments. In addition, based on the published protocol [14], a parallel desalting method is introduced to speed up peptide desalting prior to LC-MS/MS.

8.2 Materials

Only HPLC- or LC-MS-grade organic solvents are used. Buffers and solutions should be prepared with HPLC grade water.

8.2.1 Chemicals and Solutions to Process Urine Supernatants for Shotgun Proteomics

1. Amicon Ultra-15 centrifugal filter (Cat. No. UFC901096, Millipore).
2. MultiScreen Ultracel-10 Filter Plate (Cat. No. MAUF01010, Millipore). 96-well V-bottom Collection Plate (Cat. No. MSCPNPP00, Millipore).
3. Bench top centrifuge (for example, Eppendorf 5810R).
4. Misonix Sonicator 3,000 Ultrasonic Cell Disruptor.
5. SpeedVac concentrator (Thermo, SPD121P, or LabConco, MO).
6. (Optional) TMN buffer: 40 mM Tris-HCl, pH 8.1, 5 mM $MgCl_2$, and 100 mM NaCl.
7. (Optional) Lysostaphin (AMBI Products; from Staphylococcus simulans), 10 mg/mL.
8. (Optional) Mutanolysin (Sigma-Aldrich; from Streptococcus globisporus), 2 mg/mL.
9. Urea buffer: 8M urea (Sigma, U5128) in 50 mM Tris-HCl pH 8.1; urea buffer should be prepared freshly for each experiment.
10. USED lysis buffer: 8M Urea, 1 % SDS, 5 mM Na-EDTA, and 50 mM DTT; USED buffer should be prepared freshly each day.
11. IAA solution: 0.05 M iodoacetamide in 50 mM Tris-HCl pH 8.1; IAA solution should be prepared freshly each day.
12. ABC buffer: 50 mM ammonium bicarbonate in water.
13. Trypsin solution: trypsin (Promega sequencing grade); concentration of stock solution at 0.1 μg/μL stored at −80 °C.

8.2.2 Chemicals and Solutions (for StageTip Desalting)

1. Empore C_{18} Extraction disks (Cat. No. 2215, 3M).
2. Adaptor: PolyLC TopTip adaptors (Cat. No. TT200HEA) or Micro Spin columns from Hoefer (Cat. No. SP-744421).
3. Bench top centrifuge (for example, Eppendorf 5415R).
4. Activation buffer: 100 % methanol.
5. Wash buffer: 0.5 % acetic acid in H_2O.
6. Elution buffer-I: 0.5 % acetic acid, 60 % acetonitrile and 40 % H_2O. Elution buffer-II: 0.5 % acetic acid, 80 % acetonitrile and 20 % H_2O.

8.3 Methods

This protocol consists of three basic steps: (1) preparation of urine concentrates and pellets, (2) processing of samples/lysates in a 96FASP plate, and (3) StageTip desalting. Completion of these experimental steps generates a peptide-enriched fraction ready for a LC-MS experiment. A flow chart of the experimental process is presented in Fig. 8.1 and described below in detail.

(*Note* the method steps described below pertain to experimental processing of urinary pellets/sediments and urinary supernatants. In each step, it is specifically noted which of the two urine fractions is processed).

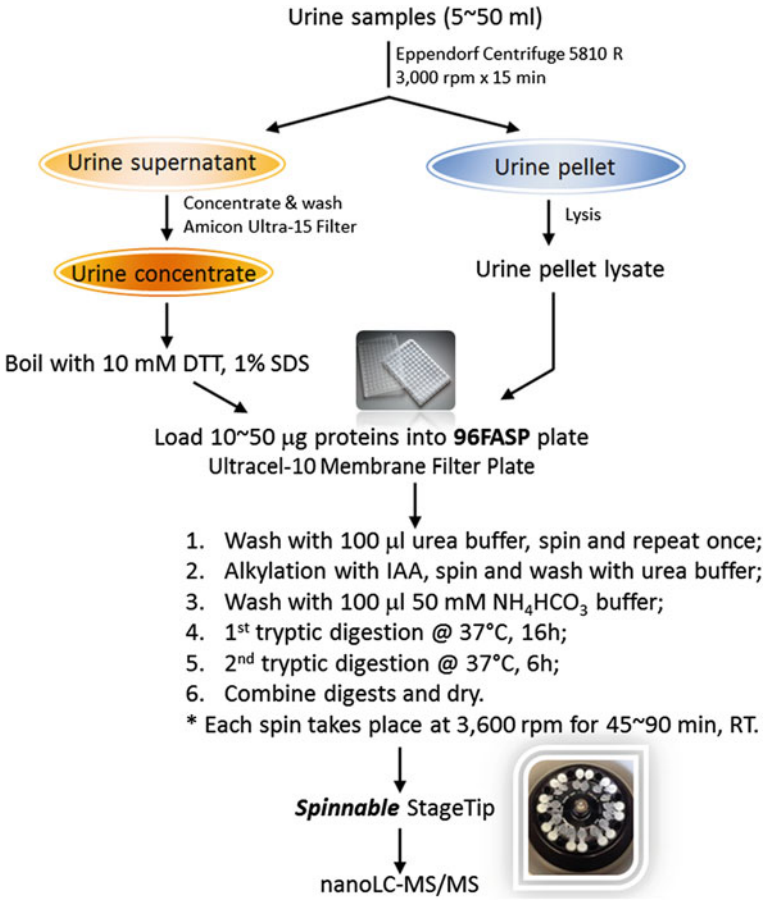

Fig. 8.1 Workflow of urine sample preparation using 96-well filter plate for shotgun proteomics. The procedures for processing both urine supernatant and pellet fractions are described. After digestion, when to clean the peptide samples, a spinnable StageTip protocol is included in the workflow. This workflow represents a high throughput approach for biomarker discovery based clinical proteomics

8.3.1 Urinary Pellet (Sediment) and Urinary Supernatant Processing

1. Urine sample centrifugation and concentration. A human subject urine specimen obtained from a clinical laboratory usually has a volume in the range of 5–50 mL. The ideal workflow starts with the processing of urine soon after collection, without intermittent freezing. A urine sample can be kept at 4 °C for up to 10 h prior to the centrifugation; however, salts may precipitate during cold storage. If it is not possible to process a urine specimen within 10 h, it can be frozen at −20 °C or −80 °C at the clinical site and shipped to the analytical laboratory when convenient. The freshly collected or thawed urine sample is equilibrated to 20 °C and neutralized to a pH of 7–8 with a 1 M Tris-HCl solution (pH 8). Urine tends to have a slightly acidic pH (6–7), but can occasionally be more acidic or basic. Neutralization at 20 °C usually dissolves crystallized uric acid and calcium oxalate, sodium urate, and magnesium ammonium phosphate salts if present.
2. Centrifuge the urine sample in a conical tube at 3,000 rpm for 15 min at 10–15 °C, carefully collect the urine supernatant, transfer to a new tube, and retain for further processing of the urine supernatant. Also recover the pellet retaining 0.5–1 mL of urine supernatant to avoid disturbing the urinary sediment (pellet).
3. *Urine pellet*: add ~10 mL phosphate-buffered saline (PBS) at 4 °C, gently shake the tube to resuspend the pellet and centrifuge for 15 min at 3,000 rpm. Discard this supernatant (the wash solution) and store urinary pellet at −80 °C or process immediately.
4. *Urine supernatant*: to concentrate the urine supernatant, transfer it to an Amicon Ultra-15 filter, and concentrate at 3,000 rpm to a volume of ~1.0 mL for 15–30 min. Multiple consecutive concentration steps may be needed if supernatant volume is larger than 15 mL and if the filtration rate is low. Wash the urine supernatant concentrate with ~10 mL PBS and spin again to reduce the volume to ~1.0 mL.
5. *Urine supernatant*: the total protein quantity in the urine concentrate is measured using Bradford Protein Assay (BioRad). To support the quantitative estimate and assess the relative abundance of albumin in the concentrate, an SDS-PAGE gel can be run.
6. *Urine supernatant*: aliquot 20–100 μg of the urinary concentrate, mix with 1 % SDS and 10 mM DTT (final concentration) and boil in microcentrifuge tube for 10–15 min at 95 °C. Cool to room temperature and the samples are ready for the 96FASP method.
7. *Urine pellet*: independent of the cellular composition of the urinary pellet, a cell disruption and protein solubilization step is required to maximize the number of distinct proteins effectively digested during the FASP analysis and to estimate the amount of total protein to be subjected to FASP and digested with a proteolytic enzyme (trypsin). To achieve effective lysis of most cells in a urinary

pellet, add USED buffer with an approximate volume ratio of 4:1 (buffer/urinary pellet). If the urinary pellet volume is very small, a minimum of 100 µL USED buffer volume should be added. Resuspend by vortexing vigorously (2 or 3 times for 30 s).

8. *Urine pellet*: pretreatment with lysostaphin and mutanolysin. Many Gram-positive bacteria have thick cell walls (e.g., various Streptococcus and Staphylococcus species). USED buffer suspension followed by sonication is not effective in lysing these cells. If the purpose of a urine pellet proteomic analysis is to investigate for UTI, possibly caused by Streptococcus or Staphylococcus species, resuspend the urinary pellet in TMN buffer in a volume ratio of 1:10 (pellet/TMN), usually less than 200 µL. Pipette the suspension up and down a few times in the microcentrifuge tube. Add lysostaphin and mutanolysin each to a final concentration of 20 µg/mL. Mix gently to homogenize enzymes and suspended cells. Incubate sample in a shaker-incubator at 37 °C for 60–150 min, while occasionally mixing to resuspend cells/cellular debris.
   Note In the case that a urinary pellet is pretreated with lysostaphin and mutanolysin, add up to 600 µL USED buffer to the lysate. Resuspend by vortexing vigorously (2 or 3 times for 30 s).
9. *Urine pellet*: using Misonex Sonicator with the water bath hooked up to it (not the probe), place urinary pellet lysates in microcentrifuge tube (from steps 7 and/or 8) in a float in the water bath filled with ice water. Set the program at amplitude 9 and sonicate for nine 45-s "on" cycles and 45-s intermittent "off" cooling cycles. The cells in a urinary pellet are disrupted further.
10. *Urine pellet*: let urinary lysate sit for approximately 5 min and spin in a bench top microcentrifuge at maximum speed (13,000 rpm) for 10 min. Transfer the lysate supernatant into a new 1.5 mL microtube. Discard pellet.
11. *Urine pellet*: take a 10 µL aliquot of the urinary pellet lysate (supernatant), mix with SDS-loading buffer (for SDS-PAGE gels) and run lysate(s) in a SDS-PAGE gel. Freeze the remaining lysate(s) at −80 °C until further use.
12. *Urine pellet*: load 2 and 4 µg BSA standards in the same gel. Coomassie Blue (CB)-G250 stain the gel followed by destaining with standard procedures [16]. From the overall CB-G250 staining intensity of urinary pellet lysate bands, estimate total protein amount per lane (lysate) compared to BSA band staining intensity.

8.3.2 96FASP Experiments for Urine Supernatant Concentrate and Urinary Pellet Lysate

Flush filter plate once with 200 µL NaOH (100 mM) and once with 200 µL urea buffer.

1. Load approximately 10–50 μg total protein (from urine supernatant concentrate or the urinary pellet lysate) into the 96FASP filter plate, and mix with 200 μL Urea buffer.
2. Spin at 3,600 rpm for around 45 min.
   Note Different brands of centrifuges may perform differently even at the same spin centrifugal force. In the hands of our laboratory, the Eppendorf 5810R centrifuge worked better than the Beckman Coulter centrifuge (Allegra 6R).
3. Add 200 μL urea buffer and repeat the spin at 3,600 rpm to concentrate until the volume in the well is reduced to ~10 μL.
4. Add 100 μL of IAA solution (final concentration 50 mM). Incubate for 20 min in the dark at room temperature, and spin for ~45 min.
5. Add 200 μL of urea buffer and spin again. The final sample volume in the filter unit should be 20 μL or less.
6. Add 200 μL of ABC buffer and spin. Repeat this step one time.
7. Add 100 μL of ABC buffer and then add trypsin in a ratio 1:50 (trypsin–-protein). Mix gently and incubate overnight at 37 °C.
   Note We notice the lid cannot seal plate very well. To keep the filter wet and avoid dry out, 100 μL of buffer should be added for overnight digestion. A parafilm could be used to seal the lid.
8. On the next day, add 100 μL of ABC buffer, spin, and collect the filtrate into a clean collection plate.
   Note Polypropylene, not polystyrene, plate was suggested by the supplier to use as collection plate in order reduce the non-specific bindings and possible sample loss.
9. Add 100 μL of ABC buffer to filter plate and trypsin (trypsin:protein: ratio is 1:50) and incubate at 37 °C for another 4–6 h.
10. Add 100 μL of ABC buffer, spin, and collect the second filtrate. Repeat this step one time.
11. Dry the peptides in a Speed-Vac. The samples are ready now for desalting with the StageTip method.

8.3.3 Peptide Desalting Using Spinnable StageTip

This method is adapted from a published protocol [14]. Several changes have been made specifically for urine sample preparation using 96FASP approach. Previously, all the StageTip steps in our lab were performed manually using syringe (e.g., 1.0 or 2.5 mL) as pressure device. Since most clinical proteomic studies may have tens or hundreds of samples involved, and 96FASP can generate many samples simultaneously, StageTip seems need to be scaled up and speeded up accordingly in order to better fit the high throughput workflow. Here, we introduce a spinnable and automatable StageTip using just bench top centrifuge. From our experience, this

method can significantly speed up the desalting steps without compromising any binding and elution efficiencies.

1. Follow the instructions in the published protocol and pack StageTips. Briefly, punch out 1–3 small disks of C18 Empore membrane using a 22 G flat-tipped syringe and ejecting the disks into P200 pipette tips. Ensure that the disk is securely and tightly (but not overtight) wedged in the bottom of the tip.
2. Place packed tips with the adaptor into 1.5-mL or 2.0-mL microtubes (Fig. 8.2). *Note* If different size or types of pipette tips are used for StageTip, one has to confirm that the tips sit in the center of the microtubes: the lower part is not too long to squeeze the tip and the upper part is short enough to close the lid of the centrifuge.
3. Conditioning I: load 100 µL activation buffer (methanol) into the tips, spin at 4,000 rpm for 1 min; conditioning II: load 100 µL elution buffer-II (0.5 % acetic acid, 80 % acetonitrile, and 20 % H_2O) into the tips, and do the same spin.
4. Equilibration: load 100 µL wash buffer (0.5 % acetic acid in H_2O) into the tips, spin at 4,000 rpm for 1 min.
5. Resuspend the dried peptides into 100 µL of wash buffer and vortex for ~10 min.
6. Binding: load 100 µL peptide solutions in the tips and spin. Reload the flow through into the tips and spin again. Repeat this binding step 2–3 times.

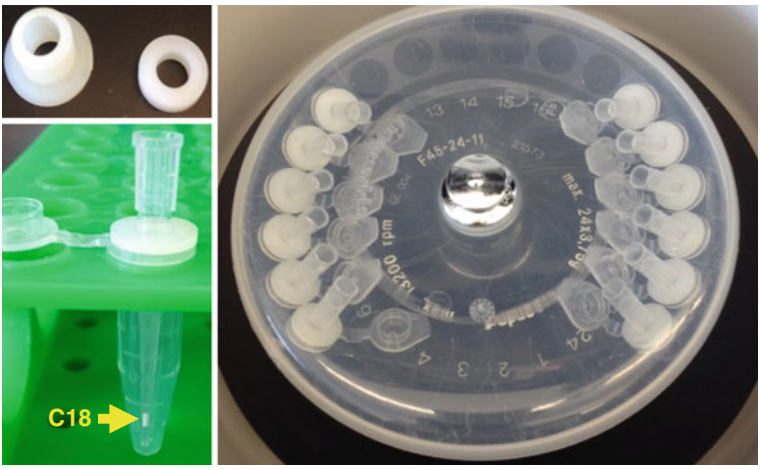

Fig. 8.2 An illustrative setup of spinnable stop-and-go-extraction tips (StageTips). Two different types of adaptors are shown, both of them can fit 1.5 and 2.0 mL microtubes perfectly. Place the packed tip (C18 disks are indicated) and the adaptor to the microtube, then all the following steps, including activation, equilibration, washing, and elution can be down by simple spins (for ~1 min) in a bench top centrifuge. In contrast to manual push using syringe, this approach can significantly speed up all the processes and can be easily scaled up as well

7. Wash: load 100 μL wash buffer and spin; repeat this step 2–3 times. The flow through during these steps can be discarded.
8. Elution: load 100 μL elution solution-I, spin, and collect elution; load 100 μL elution solution-II, spin, and collect elution in the same tube; repeat elution II one time.
9. Dry the peptide elutes in Speed-Vac, then store at −80 °C, or resuspend with HPLC buffer for immediate LC-MS/MS analysis.

Note While this chapter was under the publishing process, a detailed protocol of spinnable StageTip was made publicly available elsewhere [22]. Please refer to the detailed procedures for further information.

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Part III
Factors That Changes Urinary Proteome

Chapter 9
Variations of Human Urinary Proteome

Yong Chen

Abstract The study on dynamic analysis of human urinary proteome is the foundation that we discriminate certain various urinary proteins as potential biomarker derived from the disease itself or normal physiological change. In our results, based on RPLC-MS/MS and spectral count to study pooled and individual urine samples and other researchers' studies, it can be known that the content of many urinary proteins maintain relatively stable. We have reason to believe that the relatively stable urinary protein is a very valuable resource as biomarkers. Many similar proteins such as prostaglandin-H2 D-isomerase and apolipoprotein D proteins have been proved our hypothesis. The following field, the number, preservation and treatment methods of urine sample, the standardization of analysis method and data processing, and suitable quantitative method, is ought to the focus of future study.

Keywords Variation · Human urine proteome

The blood and urine composition remains on the correlation because of the renal filtration, and human urinary proteome analysis is a convenient and efficient approach for understanding disease processes affecting the kidney and urogenital tract.

During the biomarker discovery, dynamic variations of the urinary proteomic compared with cerebrospinal fluid and tissue samples are a more important issue. The changes in urine on a certain extent reflect the physiological status in the body. The urinary proteins list can be diversified by diet, medicines, daily activities, menstrual cycle, and other physiological conditions as well as environmental factors such as temperature and humidity. The range of urinary proteomic determines whether it has enough stability as clinical biomarker.

There are hundreds of proteins which were found in the human urine proteome at beginning. Our understanding of normal urinary proteome has been further comprehensive with the technique diversification of proteomics research; especially,

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high-resolution mass spectrometer was employed. A total of 1,543 proteins in urine were identified from ten healthy donors by a linear ion trap–Fourier transform (LTQ-FT) and a linear ion trap–orbitrap (LTQ–orbitrap) mass spectrometer [1]. A total of 2,362 proteins were obtained in normal urine, by combination of various pre-separating means, such as ultracentrifugation, gel separation, ion exchange chromatography, and reverse-phase chromatography [6]. A considerable number of proteins identified especially trace proteins were analyzed by independent research. The difference comes possibly from the various analysis strategies, different samples, and nonstandard data processing, but it verified objectively the huge variability of urinary proteome. Therefore, it is important and necessary for the clinical diagnosis to standardize the sample preparation, mass spectrometry analysis, and data processing [2]. We recommend that researchers pay more attention to variability of the urine proteome in current and future study, especially quantitative analysis.

"**Population proteomics**" **can be used to study urinary variability**. It focuses urinary proteome on the population level and gives us the better understand the diversity of proteins in human urine [9, 10]. Population proteomics concerns the change of different protein individual in the population on the specific structure and the number of the protein. In the clinical proteomics study, the introduction of the population proteomics concept can be understood as the abundance change on different conditions or time points for specified proteins. For the existed clinical diagnosis biomarkers, its abundant change is the result of the normal physiological state changes, but pathological condition is not real cause! Due to the huge variation and individual feature of normal urine, the stable urinary proteins those have significantly qualitative and quantitative difference between pathological and physiological condition may have greater chances to serve as potential urinary biomarkers. For example, kidney damage prostaglandin-H2 D-isomerase was considered as relation of hypertension and diabetes induced kidney injury [3, 4]. Pancreatic ribonuclease and alpha-1 acid glycoprotein 1 are seen as potential diabetic nephropathy biomarkers [5]. Apolipoprotein D that involved in fat metabolism in the body was associated with a various tumors, and expression level was significantly reduced in hepatoma cells [13], and the abundance of Apolipoprotein D proteins also exhibits fluctuation in different phase in prostate cancer [11]. Obviously, the prostate-specific antigen as prostate cancer biomarker can be detected in almost all the men urine. Thus, the expression profile of normal urinary proteome, especially the relatively stable members, was ought to analyze quantitatively in the first place. It is the foundation that we discriminate certain various urinary proteins as potential biomarker were derived from the disease itself or normal physiological change. Therefore, we believe that the relatively stable urinary protein is a very valuable resource as biomarkers.

In our previous study, RPLC-MS/MS and spectral count as the semi-quantitative analysis were used to study human urinary proteome variation by pooled and individual urine samples. Five types of pooled urinary samples (first morning void, second morning void, excessive water drinking void, random void, and 24-h void) collected in 1 day from six volunteers (three males and three

females) were used to analyze the urinary proteome overall intraday variations. Six pair first morning urine collected on day 0 and day 7 from above six volunteers were utilized to study inter-day, inter-individual, and inter-gender variations. A total of 31 common proteins were detected in all urinary samples, and their CV about spectra count changes from 35.3 to 97.3 %, the median CV of 59.8 %. Spectra count between the minimum and maximum number was six less than 5 times, ten 5–10 times, and fifteen more than 10 times. The intraday, inter-day, inter-individual, and inter-gender variation results showed 50 % of proteins were found in various samples and more than 40 % of these proteins whose spectral count variation was less than two times. Based on the proteomics data of urine from six volunteers, the intraday variation is less than the inter-individual variation. Inter-gender stability is the worst [12]. These results suggest that the content of many urinary proteins maintain relatively stable.

The 20 urine samples from volunteers (10 males and 10 females) were collected, respectively, by Molina et al. [7]. The occurrence of the 910 identified spots was analyzed throughout the gels and represented in a virtual 2D gel. Sixteen percent of the spots were found to occur in all samples and 23 % occurred in at least 90 % of urines. About 13 % of the protein spots were present only in 10 % or less of the samples, thus representing the most variable part of the normal urinary proteome. The quantitative analysis data of stable proteins change in the large range. Furthermore, the content of "public" urinary proteins was more than "private" proteins.

In the similar studies, Adachi showed by LC-MS that the urinary proteome of a single person comprises a major set (61.9 %) of proteins that is also found in a pool of 9 urines [1]. By LC-MS/MS label-free quantification analysis on an LTQ–orbitrap without prefractionation, Nagaraj characterized the urinary proteome of seven normal human donors over three consecutive days [8]. A total of 600 proteins were identified, and 500 proteins were readily detectable in all studied individuals. Inter-day variability was markedly higher with a CV of 0.48, and the overall variation of the urinary proteome between individuals was 0.66. These results confirm that small amounts of specific proteins coexist with the large number of common proteins in normal human urine. From the methodological point of view, the 24 % common proteins was detected by 2D gel [7], and more than 60 % of the common protein was identified by LC-MS methods [1, 8].

In the case of few studies on dynamic analysis of human urinary proteome, there is a large difference and it is difficult to compare with each other. The complexity and variability of urinary proteome is also a very important reason in addition to the technical limitations of existing proteomic methods. The number, preservation and treatment methods of urine sample, the standardization of analysis method and data processing, and suitable quantitative method especially all seriously affect the quality of the final results. Meanwhile, it is the big bottleneck for high-throughput and quantitative analysis of a large number of clinical urine samples in a short time. It can be predicted that the results from urine samples of different race, gender, age, geography, lifestyle, and collection time will be more theoretical and practical value.

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Chapter 10
Evolution of the Urinary Proteome During Human Renal Development and Maturation

Zi Wang and Mingxi Li

Abstract Renal development process in human is divided into 3 successive stages: pronephros, mesonephros, and metanephros. The tubules continue to mature for 1–2 year after birth. Research of urinary proteome during human renal development is still lacking. Most urine proteome studies focus on postnatal renal maturation period. A comparison between full-term infant and adult urinary protein pattern identified 648 infant-enriched protein spots, of which most were involved in cellular turnover and metabolism. The study of preterm infant urinary proteome compared with term infants suggests elevated IGFBP-1, IGFBP-2, and IGFBP-6, monocyte chemotactic protein-1, CD14, and sialic acid-binding Ig-like lectin 5 during nephrogenesis. Research in several congenital kidney and urinary tract anomalies, ureteropelvic junction obstruction and autosomal dominant polycystic kidney disease, has discovered novel biomarkers, which may help to imply the mechanisms underlying inherited disorders. Future exploration of urinary proteome evolution during renal maturation is needed and will help to find novel biomarkers specially suiting pediatric renal diseases.

Keywords Renal development · Urinary proteome · Biomarkers · Ureteropelvic junction obstruction · Autosomal dominant polycystic kidney disease

10.1 Introduction

Discovery of novel therapeutic targets and strategies to slow or reverse kidney injury process require an understanding of the molecular mechanisms that underlie kidney development [12]. In the past few decades, researchers mainly rely on

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molecular and biochemical techniques to explore individual genes and molecular pathways involved in normal renal development. The information has reinforced our understanding of different developmental disorders such as renal agenesis and renal dysplasia.

As a newly emerged discipline, urinary proteome analysis has been extensively applied to the discovery of diagnostics and prognostic disease biomarkers. In humans, while all segments of the nephron are present at birth, maturation of the tubule continues in the postnatal period [3]. Although there is a lack of enough literature toward the research of the urinary proteome during renal development, previous work on normal rat urine has demonstrated that the urinary proteome undergoes significant changes from birth (0 days) to adulthood (>30 days) [10].Thus, the human urinary protein patterns may change over time during late nephrogenesis and early postnatal renal maturation. Analysis of urinary proteins will offer a unique window into dynamically changes in renal developmental processes.

10.2 Human Renal Development and Maturation

Like other vertebrates, human kidney derives from the intermediate mesoderm of the urogenital ridge, a structure along the posterior wall of the abdomen in fetus [18]. Three successive stages, the pronephros, the mesonephros, and the metanephros, are gone through and mature kidney eventually emerges. From the beginning of pronephros development at 22-day gestation to the completion of nephrogenesis and tubulogenesis at 32- to 36-week gestation, full-term infants will get a complete endowment of nephrons, varying widely from 250,000 to 1.8 million per kidney, and do not form new nephrons after birth [8, 16].

Up to this point, the whole process is defined as renal development, of which metanephros transformation attracts the most research interest. From 5 week, a series of reciprocal inductive interactions occur between metanephric mesenchyme (MM) and epithelial ureteric bud (UB). Upon invasion of UB into the MM, signals from MM cause UB to undergo dichotomous branching, giving rise to the urinary collecting system. MM is also induced by the signals back from UB to condense along the surface of UB, fulfilling the mesenchymal–epithelial transition [12]. Simultaneously, interactions between epithelial and mesenchymal cells lead to a coordinated development of multiple highly specialized stromal, vascular, and epithelial cell types [15]. Previous molecular studies have implied many growth factors and signal pathways are involved in this process.

Tubules of neonatal kidney continue to maturate for an additional 1–2 year in the postnatal period to achieve the adult glomerular filtration rate, which is approximately 50 times that of neonatal kidney [1]. Some nephron segments change in abundance of transporters, while others change in transporter isoforms, paracellular permeability, or intracellular signaling that regulate the transporter [3]. All these mechanisms work together to achieve the expansion of glomerular filtration function.

10.3 Proteomic Analysis of Human Renal Development and Maturation

Urinary proteome can change under pathological conditions or alter because of normal physiologic variations. However, to date, few studies have examined the effect of normal renal development and maturation process on complete urine protein expression. Obviously, the bottleneck for conducting developmental urine proteome research is the basic fact that researchers cannot even obtain qualified urine samples before the full completion of glomerulogenesis and tubulogenesis. Currently, most published urine proteome studies focus on postnatal renal maturation period.

Froehlich et al. [7] published their work in 2013. Their group compared the urinary proteome of 6 healthy adult males (mean age: 31.7 years) with 6 healthy full-term infant males (mean age: 1.0 year) and identified three datasets. Seven hundred and eight proteins were commonly identified in both cohorts; 228 proteins were detected only in the adult samples, and 648 proteins were identified exclusively in infants. Of the 708 commonly presented proteins, 136 were significantly enriched in urine from adults and 94 were significantly enriched in urine from infants. Using gene ontology, it turned out that infant-enriched or infant-specific subproteome (743 proteins) had an over representation of proteins that are involved in translation and transcription, cellular growth and metabolic processes. In contrast, the adult-enriched or adult-specific subproteome (364 proteins) showed an overexpression of proteins involved in immune response and cell adhesion.

This study suggests cellular turnover and metabolism are increased in infants, in accordance with the postnatal tubular maturation theory. Further explorations are needed to identify biomarkers associated with renal maturation. Moreover, these data highlight the importance of age matching in urinary proteomics.

Due to the technical bottleneck mentioned above, there is only one study focusing on the renal development in fetus up to now, which was conducted by Charlton et al. [4]. They collected urine samples at birth and over 12 month from preterm (33–35 week) and term (38–40 week) infants. Utilizing a combination of G2000 antibody array and enzyme-linked immunosorbent assay, preterm infants at birth were found to have relatively elevated levels of insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2 and IGFBP-6, monocyte chemotactic protein-1, CD14, and sialic acid-binding Ig-like lectin 5 (Siglec-5). Elevated levels of multiple members of the IGF family support the critical role for this family of growth factors during nephrogenesis, which is consistent with the results of previous animal studies [5]. MCP-1, CD14, and Siglec-5 are considered markers of inflammation and were unanticipated findings.

As nephron formation continues until 36 week gestation, these 33- to 35-week preterm infants could possibly offer biomarker clues for renal development. Yet, it should be noted that variations seen in the markers may also represent changes in adaptations to extra-uterine environmental stresses that are related to preterm delivery, which is actually abnormal situations.

10.4 Biomarker Discovery from Urine Proteome in Congenital Anomalies of Kidneys and Urinary Tract

Studies of urine proteome profiles under the pathological renal developmental processes may help to imply the mechanisms underlying inherited anomalies of kidneys and urinary tract and discover novel biomarkers for clinical diagnoses and prognosis evaluation. Of this recently emerged research area, the most prominent researches are as follows:

1. **Ureteropelvic junction obstruction (UPJO):**
   UPJO occurs during early kidney development and affects renal morphogenesis, maturation, and growth [19], which is the most common prenatally detected disease leading to hydronephrosis [13]. Previous studies showed obstruction may affect the formation of nephrons. Although the decreased obstructed kidney function later normalizes, preexisting structural changes may in later years reduce the functional capacity of the kidney [6]. Researchers are challenging to distinguish those patients with severe diseases needing urgent surgical intervention out from others under rather stable conditions by utilizing urinary biomarkers. Previously, investigators have examined the cytokines already known to be up- or downregulated in UPJO or other nephropathy to find potential urinary biomarkers. Several widely known biomarkers screened out in this way are summarized in Mia Gebauer Madsen et al's review published in 2010 [11]. They are transforming growth factor β1 (TGF-β1), N-Acetyl-β-D-glucosaminidase (NAG), monocyte chemotactic peptide-1 (MCP-1), epidermal growth factor (EGF), and endothelin-1 (ET-1) (Table 10.1). Some of these biomarkers have also been tested in clinical studies.
   Recently, Hrair-George O. Mesrobian et al. obtained urine specimens from 21 healthy infants with normal maternal/fetal ultrasound and 25 infants with grade IV unilateral ureteropelvic junction obstruction. Samples from the 2 groups were subjected to liquid chromatography/tandem mass spectrometry analysis. They found 31 proteins significantly different in abundance at 1 to 6 months and 18 at 7 to 12 months compared to age-matched controls. All of the 5 biomarkers in Table 10.1 were observed with the notable exception of TGF-β1 [14]. This study utilizes the most advanced urinary proteome analysis technology to find more information about specific proteins and peptides in UPJO, which may allow for more accurate diagnosis and disease stratification. Moreover, these dynamically changing protein profiles between UPJO and control groups also provide clues into the pathological mechanism underlying clinical manifestation.

2. **Autosomal dominant polycystic kidney disease (ADPKD):**
   ADPKD is an inherited disorder affecting 1 in 1,000 people and responsible for 10 % of cases of the end-stage renal disease (ESRD) [2]. The disease is caused by mutations in the PKD1 (85 % of cases) or PKD2 gene (15 % of cases). The precise processes leading to cyst formation and loss of renal function remain

Table 10.1 Potential urinary biomarkers in prenatally diagnosed unilateral hydronephrosis [11]

Urinary biomarkers	Localization in the kidneys	Function in the kidneys	Level in the urine from children with UPJO
TGF-β1	Renal tubular epithelial cells, macrophages, and interstitial fibroblasts	The main modulator of the healing process after tissue injury	Increased
NAG	Renal tubular epithelial cells	An indicator of tubular damage	Increased
MCP-1	Renal tubular epithelial cells	Chemotactic and activating factor for monocytes	Increased
EGF	Renal tubular epithelial cells	Mediator of normal tubulogenesis and tubular regeneration after injury	Decreased
ET-1	Glomeruli and inner medullary collecting ducts and in the endothelium of renal vessels	Endogenous vasoconstrictor	Increased

incompletely understood. Early diagnosis would be of benefit for efficient planning of therapy. Kistler [9] and other colleagues published their results in 2009. Using capillary electrophoresis and mass spectrometry, they analyzed urinary samples from 17 ADPKD patients and compared with 86 samples from age- and sex-matched apparently healthy controls. After a series of selecting and eliminating procedures, 38 proteins were eventually identified as biomarkers, most of which were collagen fragments. This suggests that there is high turnover of extracellular matrix proteins. Uromodulin peptides, previously implicated in tubular injury, were also found in the urine specimens. A support vector machine (SVM)-based model was then created by combining these 38 biomarkers and including additional controls to enable high specificity. This model applied to an independent masked dataset of 24 cases and 35 healthy controls discriminated ADPKD from controls with 87.5 % sensitivity and 97.5 % specificity (AUC: 0.95). Moreover, the model remained with a high sensitivity and specificity when additionally tested in normal controls, patients with different chronic renal diseases, with bladder cancer, with renal cell cancer and elderly group (aged >60).

Although the diagnosis of ADPKD is in most cases easily established based on an age-dependent cystic renal phenotype and a positive family history [17], there is considerable inter- and intrafamilial variability in the rate of progression to kidney failure. Physicians could utilize this technique of urinary proteome to select patients with rapidly progressive disease for more radical treatments, while avoiding exposing patients with mild disease to expensive and unnecessary therapies with potential side effects.

10.5 Conclusions and Future Place

Urinary proteome analysis has been increasingly investigated to provide promising results concerning molecules participating human renal development, adding to our understanding of the mechanisms and pathophysiology underlying diseases. We expect more determination of important protein elements in signaling pathways. Developmental urine proteome is especially widely applied in investigating biomarkers of congenital renal anomalies. Since differences of urinary proteome patterns exist among different age groups, a deeper exploration of urinary proteome evolution during renal maturation will help to find novel biomarkers specially suiting pediatric renal diseases. Future contributions are still needed to better understand the series of events culminating in the formation of mature metanephros in humans. Emerging knowledge in this area will link top basic research to clinical setting in the coming years.

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Chapter 11
Hormone-Dependent Changes in Female Urinary Proteome

Annalisa Castagna, Sarath Kiran Channavajjhala, Francesca Pizzolo and Oliviero Olivieri

Abstract Human urine represents a good source for proteomic research for clinically related studies as it can be collected and processed easily and can give information about kidney-related mechanisms. Little is known about the urinary proteomic changes resulting from physiological (normal), pathological, or environmental variations, and there are few reports on hormone-related modifications of urine proteome. In our study, we highlighted the variations of urinary proteins associated with menstrual cycle or estro-progestin pill in females. We also described an association between some urinary proteins and the renin–angiotensin–aldosterone system, which might help to improve the understanding of physiological and pathological processes when a gender-specific pattern such as the menopause-related hypertension or eclampsia is evident. We therefore support the usefulness of urinary proteomics as a valuable tool for clinically related study as it can provide information on candidate biomarkers which, in turn, need to be confirmed by multiple approaches before the use in a clinical setting.

Keywords Urinary proteomics · Female hormones · Hypertension · Renin–angiotensin–aldosterone system

11.1 Introduction

Urine is one of the most attractive bio-fluids for clinically related proteomic studies due to its intrinsic properties: it retains information from the kidney through the physiological process of protein secretion from tubular cells along all the nephron and the genitourinary tract, and from more distant organs (through its circulation origin), it is easy to collect, even repeatedly and prospectively, it's stable and relatively less complex (in respect to other blood derived body fluids) [9, 19]. The

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urinary proteome, in fact, retains information from either local or distal proteomic changes, and its content could be largely affected by physiological (normal), pathological, or environmental variations [15]. Quite recently, several reports have illustrated some aspects of the normal urinary proteome, either from a methodological point of view [31, 32], or in an attempt to highlight differences in infant–adult male urine [11] and changes between adult and old general population [3]. Despite a few exceptions, proteomic studies on human subjects concern individuals of both sexes even if the gender influence in certain pathologies is a recent matter of debate. Female urinary proteomics, and specifically the influence of hormones on female proteomics, was investigated in healthy subjects, by our group [6], and in pregnant women, by others [4, 5, 7, 20, 40]. Pregnancy status could be considered as a particular hormonal state involving complex physiological mechanisms. Urinary proteomic reports in pregnancy were mainly focused on complications of pregnancy such as preeclampsia [4, 6, 7] and other pregnancy-related hypertensive disorders [20]. These studies were conducted by using a mass spectrometry-based approach and illustrated a set of putative biomarkers useful for prediction and diagnosis of preeclampsia.

In addition, a very recent report focused on an interesting aspect of proteomics, a posttranslational modification (i.e., phosphorylation) [40]; these authors describe a comprehensive urinary proteomic profile of healthy women before, during, and after delivery, giving information on the phosphoproteins that are modulated in pregnancy. This study could be very useful for the understanding of the mechanisms involved in the upkeep of a healthy and uncomplicated pregnancy.

On the other hand, urinary metabolomic research in the field of hormone-related alteration is also gaining importance [27, 36]. It is worth mentioning that, concerning disease related to hormones, many diverse proteomic studies have been conducted on cellular models or tissue [13, 30, 38] among the very recent ones.

Hormones can be of natural origin, such as the physiological modulators of human body functions, or pharmacological products; in the case of females' subjects, sexual cycle-related changes are due mainly to two natural hormones that modulate estrogen and progestogen secretion: follicle-stimulating hormone (FSH) involved in the follicular phase, and luteinizing hormone (LH) involved in the luteal phase. Estrogens and progestogens are also the two pharmacological components of oral contraceptives (OC), widely used by the female population and substantially well tolerated. However, it is important to observe that despite the reduction of undesirable side effects due to the introduction of low-dose combination and progestogen-only pills [14], OC therapy is still associated with hypertensive disease [8, 34], and uncontrolled hypertension remains as a relative contraindication for its use. Moreover, there is a widely recognized association between hormonal therapy (contraceptive or hormonal replacement in menopause) and the burden of hypertension in woman [2]. In detail, female hormones were proved early on to presumably activate the renin–angiotensin–aldosterone system (RAAS) [23], leading to blood pressure (BP) increase. The RAAS regulation is particularly important in some forms of hypertension, such as primary aldosteronism (PA), in which this system is structurally altered.

At present, PA is recognized as the most frequent cause of secondary hypertension, with an incidence varying from 5 to 15 % among hypertensive patients [10]. In PA hypertension, the screening test is represented by aldosterone-to-renin ratio (ARR). Once the result of the screening test is positive, PA diagnosis needs further confirmation to document the autonomous production of aldosterone [12]. The most widely used confirmatory tests are intravenous saline load (ivSLT), oral saline load, and fludrocortisone suppression test [12]. All these tests are time-consuming and expensive, and potentially burdened by the risk of uncontrolled hypertensive peaks [10], so they have to be reserved for carefully selected patients. In different surveys, the percentage of negative confirmatory tests after a positive ARR screening varies from 30 to 50 % [12]. Thus, factors interfering with ARR diagnostic accuracy should be recognized and if possible avoided. We documented an activation of the RAAS in the luteal phase, that is, higher renin and aldosterone values but unchanged ARR in follicular and luteal phases of the menstrual cycle, of healthy normotensive women [26]. Furthermore, we demonstrated that oral contraceptive therapy influences ARR determination in normotensive women [26], with higher ARR values in the luteal phase. This means that endogenous hormones and OC can change the levels of some hormones that are used as tests for pathology screening, leading to false positives and increasing the problem of accuracy.

The demonstration that ARR values are affected by endogenous and exogenous female sex hormones in normotensive individuals suggests the possibility that the initial ARR determination in the workup of hypertensive patients may be sufficient in male patients, but that it should probably be repeated in women. For the same reason, oral contraceptives should be included in the list of drugs to be withdrawn before ARR measurement [26].

Moreover, aldosterone, besides being involved in PA, is the principal human mineralocorticoid hormone, increasingly recognized as playing a significant role in the pathophysiology of renal and cardiovascular disease states.

Although attention has been devoted recently to non-renal action of aldosterone, unfavorable hormone-related effects are traditionally ascribed to salt and water retention at renal level and the relative consequences on extracellular fluid volume and blood pressure. Aldosterone increases the rate of sodium (Na) reabsorption across epithelial cells of the distal nephron by increasing Na transport through the epithelial Na channel (ENaC), the principal physiological target of aldosterone action [29]. In particular, an aldosterone-driven pathway leads to the activation of the ENaC at the distal tubule and collecting duct level, eventually promoting cell Na entry for potassium (K) exit in equimolar proportions. The ENaC is a membrane complex localized not only in the kidney but also in several other tissues and organs involved in Na maintenance (i.e., airways, skin, colon, and salivary glands). The channel is composed of three homologous subunits (α, β, and γ) [1] that are functionally complementary in activating the ENaC by a sequential mechanism of cooperation. In recent years, much attention has been devoted to proteolytic mechanisms able to activate the ENaC by removing inhibitory peptides on the β or γ chains. One of the most interesting proteins investigated in this context is prostasin, a 40-kDa serine protease first detected in the prostate gland and seminal fluid.

Prostasin activates ENaC when expressed in oocytes, and the addition of aldosterone to cultured mouse collecting ducts increases ENaC activity by increasing prostasin secretion and mRNA and protein expressions [24].

Aldosterone induces the activation of prostasin and, in cooperation with other protease(s), such as furin, cleaves (at one or more sites) the γ-subunit of ENaC, contributing to the removal of an inhibitor peptide and increasing the probability of the channel opening. Prostasin is also of interest because it is a glycosylphosphatidylinositol (GPI)-anchored protein, secreted in extracellular fluids such as urine, and the amount released in urine seems to be grossly proportional to the extent of ENaC activation [24]. As regards the influence of hormones on prostasin modulation, it has recently been reported that prostasin is expressed at a relatively high level in human placenta trophoblasts in the early weeks of pregnancy [21], further supporting a possible correlation with sex hormones. As physiological cyclic variations of female hormones alter Na balance and affect salt retention, we hypothesized that urinary prostasin might be accordingly modulated during different phases of the menstrual cycle and/or after OC therapy [25].

11.2 Changes in Female Proteome Due to Cycle-Related Hormones and OC Intake [6]

A group of healthy young women (mean age 28.5 ± 7.9 years) was enrolled for the study, urine samples were taken, and clinical parameters related to RAAS (blood pressure (BP), urinary Na, plasmatic K, aldosterone, renin, FSH, LH, estradiol, and progesterone) were recorded at follicular and luteal phases and after OC intake. Urine samples were processed and pooled for the subsequent proteomic analysis. A classical proteomic approach based on 2D-PAGE was applied to study the urinary proteome of female subjects at different hormonal states, aimed at the identification of proteins undergoing modulation associated with female hormone variations and, in turn, possible connection with sexual hormones to RAAS. Regarding this analysis, samples corresponding to mid-cycle phase (G1), luteal phase (G2), and after two months of contraceptive therapy with a third-generation OC pill (G3) were analyzed; for a complete overview of the mechanisms involved, a fourth comparison was performed, considering G1 and G2 as a whole (G = "untreated samples") *versus* G3 ("treated samples"). Figure 11.1 reports the standard maps (as obtained by PDQuest software) for the four comparisons investigated (G analysis). The software analysis gave an output of 40, 17, 34, and 24 differentially modulated protein spots in the comparisons G1–G2, G2–G3, G1–G3, and G–G3, respectively. After software analysis, the differentially expressed proteins were excised from the gels and analyzed by MS/MS for identification. The differential proteomic analysis performed allowed the detection of many proteins related to the physiological changes or induced by the OC treatment as illustrated in Table 11.1.

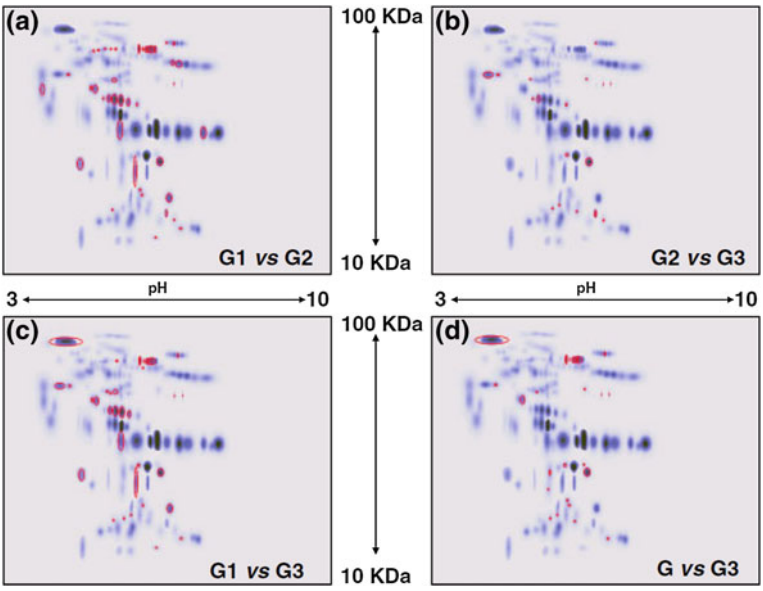

Fig. 11.1 Standard maps obtained with the analysis software PDQuest for the four comparisons investigated. The differentially expressed spots are shown as *red circles* **a** in G1 versus G2; **b** in G2 versus G3; **c** in G1 versus G3; and **d** in G versus G3 comparisons. From Ref. [6] with permission

Regarding the molecular function of the modulated proteins, it is worth noting that the majority could be classified as protein binding, endopeptidase inhibitors, ion binding, or other, while according to their cellular localization, we found that most of the identified proteins were secretion proteins. Others were derived from cytoplasm, lysosomes, or extracellular proteins.

We did not observe severe modulation in protein "expression" as just one protein in one comparison was found so extremely modulated as to be considered newly expressed (cystatin A), but the observed changes are interesting in any case in terms of their association with RAAS activation. The clinical parameters annotated in the three phases under study showed a particular trend: gonadotropin and progesterone were different along the phases of the cycle, and in parallel, renin and aldosterone were increased in the luteal phase. After OC intake, systolic blood pressure (SBP) and diastolic blood pressure (DBP) showed an increase, while FSH, LH, progesterone, and estradiol were lower during the therapy in agreement with the inhibitory effect of OCs on gonadotropin regulation. Aldosterone decreased after OC therapy, whereas urinary sodium excretion and renin increased.

Furthermore, as the G1 and G2 phases taken together could mimic a normal situation, we focalized on the comparison taking G1 + G2 as a whole (G) versus G3. The proteins found down-regulated were perlecan, aminoacylase 1, fatty acid-binding protein 5, S100 calcium-binding protein A8, and serum albumin, while the

Table 11.1 List of proteins identified by MS/MS analysis

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Alpha-1-micro-globulin/bikunin	AMBP	403	P02760	gi|4,502,067	2	142	/39,886	/5.95	down 2,7			
Aminoacylase 1	ACY1	6,401	Q03154	gi|4,501,901	9	332	/45,856	/5.77		down 2,13		down 1,78
Annexin A3, iso-form CRA_b	ANXA3	5,302	P12429	gi| 119,626,228	7	236	/39,845	/6.68	up 3,22	down 2,5		
Clusterin	CLU	2,301	P10909	gi|338,305	3	151	/36,997	/5.74	up 3,53		up 4,31	up 2,08
Clusterin	CLU	2,302	P10909	gi|178,855	7	79	48,772	6.27	up 4,16	down 2,78		
Clusterin	CLU	2,402	P10909	gi|178,855	5	197	/49,342	/6.27	up 2,89		up 2,55	
Clusterin	CLU	3,302	P10909	gi|178,855	6	246	/48,772	/6.27	up 3,17	down 1,78	up 1,76	
Clusterin	CLU	3,304	P10909	gi|338,305	3	184	/36,997	/5.74	up 2,43		up 1,69	
Clusterin	CLU	3,401	P10909	gi|178,855	3	101	/49,342	/6.27			up 7,19	
Clusterin	CLU	4,302	P10909	gi|178,855	2	107	/49,342	/6.27	up 2,67		up 1,97	
Clusterin	CLU	4,304	P10909	gi|178,855	2	98	/49,342	/6.27	up 3,64		up 1,71	
Beta-2 microglobulin	B2 M	7,002	P61769	gi|34,616	3	169	/12,905	/5.77	down 3,33	up 1,77		
Complement component C4B	C4B	2,403	P0C0L5	gi|187,771	1	99	/40,795	/5.19		up 3,91		
Cystatin A	CSTA	4,104	P01040	gi|4,885,165	2	96	/11,000	/5.38		new induced	up 9,41	up 15
Fatty acid-binding protein 5	FABP5	5,102	Q01469	gi|4,557,581	4	135	/15,155	/6.60	down 1,89			down 1,69
Fatty acid-binding protein 5	FABP5	5,104	Q01469	gi|4,557,581	4	135	/15,155	/6.60	down 1,61		down 2,78	down 2,13

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Fatty acid-binding protein 5	FABP5	6,104	Q01469	gi|4,557,581	2	84	/15,497	/6.60	down 1,54		down 1.89	
Gelsolin	GSN	1,403	P06396	gi|17,028,367	3	169	/31,052	/4.85	up 3	up 2,5	up 7,56	up 4,07
Gelsolin	GSN	3,403	P06396	gi|17,028,367	3	147	/31,052	/4.85	up 2,84		up 3,36	
Perlecan	HSPG2	5,202	P98160	gi|11,602,963	4	208	/477,024	/6.03		down 2	down 2,78	down 1,89
Perlecan	HSPG2	6,103	P98160	gi|11,602,963	2	141	/477,024	/6.03	down 2,38	up 6,20	up 2,60	up 3,95
Perlecan	HSPG2	6,202	P98160	gi|184,427	3	72	/481,880	/6.05				down 3
Ig heavy chain variable region	IGHV3-23	7,506		gi|112,695,045	2	75	/10,785	/9.06	up 2,38			
Ig heavy chain variable region	IGHV3-23	7,507		gi|112,695,045	2	75	/10,785	/9.06	down 1,67			
Ig kappa light chain 1	IGKV	4,206		gi|4,176,418	6	397	/23,690	/6.92	down 1,89		down 2,22	
Ig superfamily, member 8	IGSF8	5,108	Q969P0	gi|16,445,029	4	206	/65,621	/8.23	down 4		down 2.38	
Mannan-binding lectin-ass. serine protease 2	MASP2	4,103	O00187	gi|3,297,879	4	180	/77,176	/5.43		up 1,83	up 2,01	up 2,1
Prostaglandin H2 D-isomerase	PGDS	8,201	O60760	gi|32,171,249	3	80	/21,299	/7.66	up 3,11			
Calgranulin A	S100A8	7,004	P05109	gi|29,888	2	89	/10,988	/9.19	down 1,70		down 2,86	down 2,1

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Calgranulin B	S100A9	5,001	P06702	gi|4,506,773	5	242	/13,291	/5.71		up 4,61		up 4,3
S100 calcium-binding protein A7	S100A7	6,003	P31151	gi|12,053,626	4	68	/10,410	/6.08	down 2	up 1,68		
Serpin peptidase inhibitor, clade B, member 3	Serpin B3	7,304	P29508	gi|239,552	3	185	/44,564	/6.35		up 2,75	up 2,67	up 3,08
Serpin peptidase inhibitor, clade B, member 3	SerpinB3	7,305	P29508	gi|239,552	4	170	/44,564	/6.35	down 1,79	up 4,18	up 2,33	up 2,11
Serum albumin	ALB	2,706	P02768	gi|3,212,456	22	1,082	/68,425	/5.67	down 7,14			
Serum albumin	ALB	2,707	P02768	gi|28,590	12	622	/71,246	/5.92	down 4,17			
Serum albumin	ALB	2,708	P02768	gi|28,592	18	914	/71,316	/6.05	down 20			
Serum albumin	ALB	3,706	P02768	gi|28,592	12	661	/71,316	/6.05	down 8,33		down 3,13	
Serum albumin	ALB	3,707	P02768	gi|28,592	17	866	/71,316	/6.05	down 16,67			
Serum albumin	ALB	5,703	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,82		down 2,44	down 1,82
Serum albumin	ALB	5,704	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,67		down 2,13	down 1,64
Serum albumin	ALB	5,705	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 2		down 3,23	down 2,22
Serum albumin	ALB	6,705	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,79			

(continued)

Table 11.1 (continued)

Protein name	Gene name	Spot no.	SwissProtTrEMBL acc. #	NCBI acc. #	No. of pep identified	Mascot score	TMr. (kDa)	PI theor.	G1 versus G2 *	G2 versus G3 *	G1 versus G3 *	G versus G3 *
Serum albumin	ALB	6,706	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,75		down 2,1	down 1,59
Serum albumin	ALB	6,707	P02768	gi|3,212,456	18	1,070	/68,425	/5.67	down 1,96		down 2,33	down 1,69
Tetranectin	CLEC3B	4,102	P05452	gi|267,108	1	60	/22,552	/5.52				up 1,66
Thioredoxin	TXN	3,103	P10599	gi|1,065,111	2	131	/11,629	/4.82	down 2.63		down 1,78	
Transferrin	TF	6,702	P02787	gi|4,557,871	8	449	/79,280	/6.81	down 3,84	up 2,33		
Transferrin	TF	7,701	P02787	gi|553,788	8	449	/79,280	/6.81	down 1,75			
Transferrin	TF	7,702	P02787	gi|553,788	8	409	/55,207	/6.00			up 3,02	up 2,11
Ubiquitin	UBB	6,004	P62988	gi| 134,105,063	3	161	/8,571	/5.73	down 1,82		down 1,67	
Uromodulin	UMOD	1,801	P07911	gi|340,166	3	165	/72,429	/4.97			up 2,46	up 1,87
Vitelline membrane outer layer 1	VMO1	1,101	Q7Z5L0	gi|32,698,964	2	155	/22,033	/4.90	down 1,89		down 1,89	
Zn-alpha2-glycoprotein	AZGP1	3,306	P25311	gi|38,026	2	73	/34,942	/5.71			up 3,36	up 3,04

Modified from Table 1 in Ref. [7] with permission

Note *: Fold of variation in expression: *increased protein* (Up = in the right-hand group), *decreased protein* (Down = in the right-hand group)

ones up-regulated were clusterin, cystatin A, gelsolin, mannan-binding lectin-associated serine protease 2, S100 calcium-binding protein A9, serpin peptidase inhibitor, clade B, member 3 (serpin B3), tetranectin, uromodulin, and Zn-a2-glycoprotein.

Interestingly, and probably due to different posttranslational regulations, some proteins were present in both groups, as either over- or under-expressed.

We then focused our attention on 3 proteins for further validation by Western immunoblotting (uromodulin, clusterin, and serpin B3) as shown in Fig. 11.2 and ELISA (serpin B3).

Uromodulin represents one of the most abundant proteins present in human urine; it is produced by the thick ascending limb of the loop of Henle of mammalian kidney, and it is a GPI-anchored glycoprotein. This protein is probably involved in many kidney pathologies such as tubulointerstitial nephritis, kidney stones, cast nephropathy, and urolithiasis [33]. In Fig. 11.2, it is possible to notice that uromodulin abundance was increased in the G3 pool, in agreement with the proteomic analysis and with observations that uromodulin could be associated with salt intake in healthy individuals [37], as after OC therapy, sodium excretion was found to be increased (G vs. G3 comparison) in our set of individuals.

Concerning the other two proteins, clusterin and serpin B3, validated by Western immunoblotting, we could confirm the trend observed in the 2D gels regarding their abundance after OC treatment (see Fig. 11.2 panels B and C). The protein clusterin is known to be involved in many biological functions, and it was previously associated with angiotensin II action (which is induced by estro-progestogen treatment) [39].

On the other hand, serpin B3, a protease inhibitor of the serpin family, was further investigated by means of a commercial ELISA kit adapted for urine analysis. Our data on individual samples provided a further confirmation of serpin B3 increase after OC treatment. Other reports in mouse described the association of serpins with angiotensin II [28] or the regulation of serpin genes by gonadotropins [22]. In addition to these data, we also observed a further increase in serpin B3

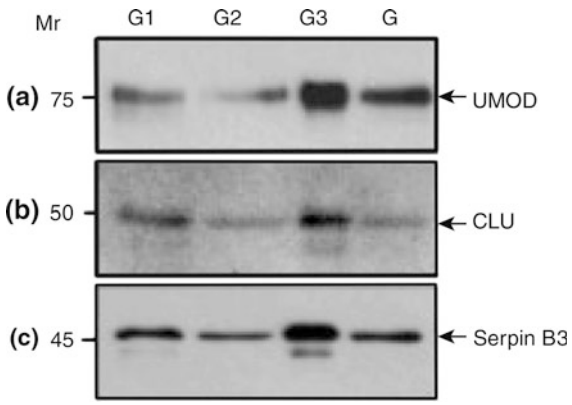

Fig. 11.2 Western immunoblotting obtained after SDS-PAGE and incubation with antibodies anti-uromodulin (**a**), clusterin (**b**), and serpin B3 (**c**). From Ref. [7] with permission

expression in a group of 5 pregnant women, with a gestational range of 20–-25 weeks, indicating a hormonal control on serpin B3 expression, or secretion, in urine [6].

Our work on urinary proteomics of healthy women enabled the identification of some proteins likely to be associated with RAAS changes induced by hormones: uromodulin, clusterin, perlecan, and serpin B3. Further investigations in the field of contraceptive- or menopause-associated hypertension could benefit from our preliminary discoveries.

11.3 Changes in A Specific Protein (Prostasin) Related to ENaC Activation in Urine of Healthy Subjects According to Different Phases of the Menstrual Cycle and/or After OC Therapy [25]

Urinary prostasin was assessed in a subset of healthy women in the same phases (G1, G2, and G3) of the comparative proteomic illustrated in paragraph 3. In fertile women, the comparison between the follicular and luteal phases confirmed the activation of the aldosterone–renin axis during the menstrual cycle, with raised luteal aldosterone and renin concentrations but without concordant modifications of their ratio (aldosterone range 167 ± 90 to 341 ± 175 pg ml^{-1}, $P = 0.01$; renin range 12 ± 8.3 to 20 ± 12.4 pg/ml, $P = 0.05$; ARR range 17.4 ± 11.3 to 20.7 ± 11, P = NS). Similar to the ARR, the urinary prostasin level did not substantially vary in the luteal compared with the follicular phase (prostasin range 2.97 ± 3.58 to 2.35 ± 1.23 nM; data not shown). After OC therapy, the aldosterone and renin values were significantly reduced (aldosterone range 341.0 ± 175.5 to 201.0 ± 99.7 pg ml^{-1}; renin range 19.7 ± 12.5 to 6.9 ± 3.3 pg ml^{-1}), whereas the ARR increased (from 20.7 ± 11.0 to 33.7 ± 18.3). Urinary prostasin also tended to increase (2.37 ± 1.27 to 4.85 ± 5.28 nM) consistent with the ARR; however, this increase was not statistically significant ($P = 0.07$; Table 11.2).

In healthy women, the prostasin level, unlike the aldosterone level, did not substantially change following the menstrual cycle phases. Therefore, although sexual hormone fluctuations are indeed able to affect aldosterone and renin concentrations, the release of prostasin in urine does not appear to be sufficiently sensitive to these variations, or speculatively, ENaC activity does not actually change in a significant way. Estro-progestinic therapy had a greater capability to alter such a balance. In fact, despite slight changes in the aldosterone and renin levels (both decreased), the ARR and the urine concentration of prostasin increased, but the increases did not reach statistical significance. Moreover, although the BP values did not reach pathological levels, they increased substantially after OC therapy and accompanied a significantly increased U-Na.

Table 11.2 Clinical and biochemical characteristics of the group of healthy women before and after estro-progestinic therapy

	Before OC therapy	After OC therapy	*P*
SBP (mm Hg)	108 ± 7,7	114 ± 11,5	0,05
DBP (mm Hg)	70 ± 5,6	73 ± 7,7	NS
Aldosterone (pg/ml)	341,0 ± 175,5	201,0 ± 99,7	0,01
Renin (pg/ml)	19,7 ± 12,5	6,9 ± 3,3	0,002
ARR	20,7 ± 11,0	33,7 ± 18,3	0,001
P–K (mmol/L	3,79 ± 0,3	3,85 ± 0,2	NS
U–Na (mmol/L)	108,8 ± 44	146,0 ± 34,8	0,014
U–prostasin (nM)	2,41 ± 1,27	4,85 ± 5,28	0,07
U–prostasin/U–Na (nM/mmol*L^{-1})	0,023 ± 0,014	0,033 ± 0,03	NS

From Ref. [25] with permission
Data are means ± SD
SBP systolic blood pressure; *DBP* diastolic blood pressure; *ARR* aldosterone-to-renin ratio (pg.mL^{-1} /pg.mL^{-1}); U-prostasin/U-Na = ratio between urinary prostasin concentration in nM and urinary Na in mmol/L

In conclusion, in healthy subjects, urinary prostasin is similarly present in both genders, and it is not affected by the different phases of the menstrual cycle. Prostasin is modulated by urinary Na, and prostasin levels appear to be correlated with the ARR rather than with individual aldosterone or renin levels. Although a urinary excess of prostasin seems to be associated with ARR, which is the hallmark of conditions such as low-renin hypertension and primary aldosteronism, the precise relationship linking the renin–angiotensin−aldosterone axis, Na, and prostasin in hypertensive patients needs to be further investigated.

11.4 Methods and Protocols

11.4.1 Healthy Fertile Women on Free and OC-Induced Menstrual Cycles

Young healthy normotensive women, with no history of disease and referred for contraceptive counseling to a single physician at the Obstetric and Gynecology Department of the University of Verona, were enrolled in the study. Urine and blood samples were collected in the follicular phase of their menstrual cycles (between 14th and 16th day), in the luteal phase (between 20th and 22nd day) and after 2 months of OC therapy. All patients were prescribed the same third-generation pill, which consists of 0.075 mg gestodene and 0.02 mg estradiol. At each of the three time points, BP was measured and the average values were recorded. The protocol was approved by our institutional review committee, and written informed consent

was obtained from each subject before initiation of the study. Systolic BP and diastolic BP were carefully measured in all enrolled study subjects. BP was evaluated twice in both arms, with the subject remaining in the sitting position for 5 min and the subject avoiding coffee intake and smoking during the preceding 30 min. Further measurements (when necessary) were taken if the differences in BP values were X5 mm Hg.

11.4.2 Urine Sample Collection and Processing

Fresh urine samples were collected in the morning, immediately chilled on ice, and processed according to the previously established protocol [24]. An aliquot of urine was examined for white and red blood cells by a urinalysis test, and the samples of individuals in whom significant microematuria and/or leukocyturia (>10–20 cells) had been detected were excluded from any further analysis. When not immediately concentrated, the processed urine was stored at −80°C until required. Protein concentration was evaluated with Bradford assay (Bio-Rad).

11.4.3 Prostasin and Serpin ELISA Assays

Urine processed as described above was processed in duplicates for both assays.

11.4.3.1 Prostasin

A competitive enzyme-linked immunosorbent assay (ELISA) test was set up by exploiting the rabbit response toward a specific, highly immunogenic, prostasin peptide (amino sequence: AHQLDSYSEDAKVSTLKDI). Polyclonal antibodies were obtained from rabbits inoculated according to the method previously described by Koda et al., and the assay was conducted accordingly [17].

11.4.3.2 Serpin B3

The quantitative measurement of serpin B3 inhibitor was taken with a commercial ELISA kit (SCCA-LISA) purchased from Xeptagen according to manufacturer's recommendations. As the kit was originally designed for serpin B3 assay in serum, several modifications were introduced to adapt the assay and to obtain reproducible and reliable results also in urine [6].

11.4.4 Biochemical Analysis

The patient blood samples were collected after an overnight fast. All of the biochemical tests were performed in the Laboratory of Clinical Chemistry of the University of Verona. Aldosterone and direct active renin levels were determined using commercially available methods (DiaSorin Diagnostics, Turin, Italy) and expressed in pg/ml.

11.4.5 Statistical Analysis

Statistical analyses were performed with SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). The quantitative values were expressed as means ± SD. When necessary, logarithmic transformation was applied to obtain a normal distribution, and the variability of the data was expressed as 95 % confidence intervals. Student's t test for paired data was performed by comparing data from the same subjects. Correlations were tested by simple bivariate correlation analysis.

11.4.6 SDS-PAGE and Western Immunoblotting

Aliquots with the same amount of total protein content (25 μg) were loaded onto 12 %T SDS-polyacrylamide gels, and 1D SDS-PAGE was performed according to Laemmli [18] as previously described [24]. After electrophoresis, resolved proteins were blotted to a polyvinylidene difluoride (PVDF) membranes (Bio-Rad) as previously described [24]. Membranes were stained with amido black (Sigma-Aldrich) for a few minutes, and unbound stains were removed with destaining buffer (25 % isopropanol, 10 % acetic acid) for 30 min at room temperature. Proteins stained in PVDF membrane were captured using Bio-Rad Chemidoc XRS Imaging System for normalization purposes. In order to prevent non-specific binding of primary antibody, PVDF membranes were blocked overnight at 4 °C with 5 % non-fat dried milk (Sigma-Aldrich) in 0.01 % Tween-20 Tris-buffered saline. Then, membranes were probed with primary antibody against clusterin, uromodulin, and serpin B3 at the appropriate dilutions (1:500; 1:500; 1:2,000) in 1 % non-fat dried milk in 0.05 % Tween-20 Tris-buffered saline for 2 h at room temperature. Unbound primary antibody was removed with 3 washes of 10 min each with 0.05 % Tween-20 Tris-buffered saline. Blots were then incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000 dilutions, GE Healthcare). The immunocomplex was detected by enhanced chemiluminescence (ECL, GE Healthcare) on X-Omat AR film (Kodak). The intensity of chemiluminescence response was measured by scanning films and processing the image using Quantity One software version 4.4 (Bio-Rad).

11.4.7 2D-PAGE

Pooled samples were used for the 2D-PAGE separations. The desired volume of each sample was precipitated overnight at 4 °C according to the protocol illustrated in Ref. [16].

The precipitated pellets were resuspended in 150 μl of 2D solubilizing solution, 7 M urea (Sigma-Aldrich), 2 M thiourea (Sigma-Aldrich), 2 % w/v CHAPS (Sigma-Aldrich), and 40 mM Tris (Sigma-Aldrich) and incubated with 5 mM tributylphosphine and 10 mM acrylamide (Bio-Rad) for 60 min at room temperature to reduce protein disulfide bonds and alkylate the cysteine thiolic groups. Later, 10 mM DTT (Sigma-Aldrich) was added in order to stop the reaction. Then, pH 3–10 immobilized pH gradient strips (IPG; Bio-Rad) were rehydrated for 4 h with 150 μl of 2D solubilizing solution (containing 2.5 mg/ml of total urinary proteins), and then, proteins were focused till the total product time x voltage applied was 25,000 Vh for each strip. The IPG strips were equilibrated with 6 M urea (Sigma-Aldrich), 2 % w/v SDS (Sigma-Aldrich), 20 % v/v glycerol (Sigma-Aldrich), and 375 mM Tris–HCl pH 8.8 for 26 min in rocker. For the second dimension, 12 %T SDS-PAGE was used, with the following protocol: 5 mA for each gel for 1 h, then 10 mA/gel for 1 h, and 20 mA/gel until the tracking dye, bromophenol blue, reached the anodic end of the gels. The protein zones were finally revealed with Sypro Ruby protein gel stain (Bio-Rad).

11.4.8 Protein Pattern Differential Analysis and Further Processing of Protein Spots (In-Gel Digestion and Peptide Sequencing by Nano-RP-HPLC-ESI-MS/MS)

Gels were scanned using a Bio-Rad VersaDoc 1,000 Imaging System. 2D gel analysis was performed by PDQuest software (Bio-Rad), version 7.3, as illustrated in Ref. [6].

Spots showing a statistically significant differential expression were carefully cut out from 2D Sypro Ruby-stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al. with minor modifications [35]. Peptide sequencing by nano-RP-HPLC-ESI-MS/MS was performed as previously described as well as protein identification using either a nanoflow HPLC system (Agilent 1,200 series) coupled with an ion trap (model Esquire 6,000 Bruker Daltonik) or a nanoflow HPLC system (Ultimate; Switchos; Famos; LC Packings) coupled with a high-capacity ion trap (model HCTplus Bruker Daltonik) [6]. Protein identification was performed by searching in the National Center for Biotechnology Information non-redundant database (NCBInr) with the Mascot program (Matrix Sciences).

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Chapter 12
Effects of Exercise on the Urinary Proteome

Maxie Kohler, Wilhelm Schänzer and Mario Thevis

Abstract Exercise-induced proteinuria has been observed and studied for more than a century. It was found that different sport disciplines alter the urinary proteome in different ways. Moderate-intensity exercise results in increased glomerular filtration, meaning that medium-sized proteins are excreted in higher amounts, while high-intensity exercise of short duration also increases the excretion of low molecular weight proteins as a result of tubular dysfunction. Exhaustive exercise may lead to the excretion of hemoglobin or myoglobin, which changes the urinary proteome considerably. Studies comparing protein maps of different sport types compared to a control group showed that quality and quantity of urinary proteins are interindividually different. In addition, urine samples collected before and after exercise exhibit substantially different protein patterns even from the same person. Therefore, further studies investigating the urinary proteome are desirable. As the variation of protein content and composition in urine are generally much higher than in other matrices, respective studies need to be well controlled and homogenous groups of volunteers should be chosen. In addition to the sport-related physiological and biochemical interest, exercise-induced protein changes also need to be considered for biomarker measurements from urine samples for kidney or other diseases.

Keywords Urinary proteome · Exercise · Biomarker · Kidney diseases

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12.1 Analysis of Proteins in Urine After Exercise

First reports about exercise-induced albuminuria are from Williams and Arnold 1899 in the context of the Boston marathon, where the amounts of urinary albumin were found to be higher after the marathon than prior to the competition [35]. Dunhill and Patterson [5] as well as Collier and Lond [4] later demonstrated that exercise-induced albuminuria is reversible and probably not a sign of an impaired kidney function or Brights' disease, but a commonly occurring phenomenon following exercise. It was also recognized already at that time that the amount of protein in urine relates to the amount of work that was done. Before these studies and the conclusion that proteinuria is a frequently observed exercise-induced and reversible reaction, Collier and Lond and several other physicians recommended athletes to cease sports and live quiet lives if protein was found in urine. The assumption at that time was that the vascular system was not strong enough, or the organs were too weak for high-intensity exercise. In the early days, protein determination measurements were taken using the nitric acid or acetic acid test and the Esbach's albuminometer [9] measuring the height of the protein precipitate.

The protein/creatinine ratio is used today as normalized value for the excreted protein amounts and allows the evaluation of spot urine samples. Children have a higher total protein concentration and protein/creatinine ratio than adults [13]. In the 1960s [24], the differentiation between glomerular and tubular proteinuria was observed [8] and Poortmans was the first to analyze different urinary proteins instead of total protein (namely tryptophan-rich prealbumin, albumin, α_1-acid glycoprotein, α_1-antitrypsin, ceruloplasmin, haptoglobin, Gc-globulin, transferrin, hemopexin, β_2-glycoprotein I, γA-globulin, and γG-globulin) before exercise and after a marathon run [28]. Urinary protein changes were monitored for different sport types, and a few studies performed proteomics for the untargeted investigation of protein alterations.

12.2 Mechanism of Exercise-Induced Proteinuria

In contrast to the wide field of kidney diseases, the impact of exercise on renal function(s) is not investigated as intensely. Nevertheless, several studies were dedicated to the elucidation of the mechanisms of post-exercise proteinuria, and an early observation in that regard was that proteinuria depends on exercise intensity rather than exercise duration [30].

The mechanisms of exercise-induced proteinuria are not entirely understood, but it is established that glomerular proteinuria appears at lower exercise intensity than tubular proteinuria. Glomerular proteinuria is characterized by the excretion of medium-sized proteins such as albumin, while tubular failure leads to increased

excretion of smaller proteins such as α1-microglobulin. At high-intensity exercise, a mixed-type proteinuria is detected with elevated amounts of small- and medium-sized proteins. Glomerular filtration is determined by the blood flow in the Bowman's capsule as well as the permeability of the glomerular basement membrane, which is defined mainly by negative charges from heparan sulfate proteoglycans [8]. Acidity changes in the circulation and therefore charge alterations of the glomerular membrane as well as proteins were discussed as one reason for increased excretion of specific proteins. A direct and exclusive connection of lactate concentration to the quality and quantity of proteinuria was excluded after lactate injection at rest did not induce proteinuria [26]. Nevertheless, there is a strong correlation between blood lactate and proteinuria. Although the mechanism is unclear, it is assumed that a decrease in charge of the glomerular basement membrane results in higher permeability and filtration rate [29]; hence, if the charge of the proteins plays a major role, the relative amounts of proteins would change with altered blood acidity. An influence of the adrenergic system was also proposed and shown by the application of an α2-adrenergic agonist, resulting in a reduced catecholamine response during exercise [31]. The renal blood flow drops as a result of renal vasoconstriction, which increases the glomerular filtration rate. It was found that prostaglandin inhibition attenuated increased exercise-induced protein excretion, but that inhibition of angiotensin-converting enzyme did not alter the protein excretion after exercise, indicating that the renin–angiotensin system regulating blood pressure as well as water and electrolyte equilibrium is not responsible for proteinuria [20]. In contrast, angiotensin II inhibition in rats attenuated proteinuria [6]. Tubular reabsorption is mainly receptor-mediated and may be saturated when glomerular filtration increases. In addition, inhibition mechanisms leading to a further increase in the excretion of low molecular weight proteins are discussed [3].

12.3 Hematuria and Myoglobinuria

Although the mechanisms of hematuria and myoglobinuria will not be part of this report, these two alterations have to be mentioned as they may significantly change the urinary proteome and lead to the detection of hemoglobin or myoglobin. Hematuria originates from mechanical stress, e.g., in long-distance runners in the foot capillaries as well as molecular mechanisms including filtration of erythrocytes in the glomerulus, decreased renal blood flow, damage to the nephrons, or dehydration increasing the molarity of the blood [3, 14, 23]. Myoglobinuria is a result of the rupture of muscle membranes from extreme exercise. In that case, myoglobin as well as hemoglobin may be found in urine [3].

12.4 Different Exercise Types and Intensities and Their Effects on Urinary Proteins

A number of different studies have been performed that analyze the effect of different types of exercise on proteinuria or specific urinary proteins. Proteinuria was found in 61 % of male and 66 % of female elite badminton players after competition. In addition, the presence of leukocytes (men = 43.5 % and women = 50.0 %) and erythrocytes in urine was investigated (men = 50.0 % and women = 21.7 %) [1]. A two-hour karate training session (elite athletes, female) did not result in elevated protein/creatinine ratios [33]. Moreover, the impact of different swimming distances (100, 600, 2,000 m) on proteinuria was assessed, demonstrating that the endurance distance yielded only elevated albumin levels, while the shorter distances caused an increase in the tubular marker ß2-microglobulin that gradually increased with increasing swim speed. Further, Poortmans found out that a 100-km run resulted in glomerular but not tubular proteinuria [27], while intermittent exercise

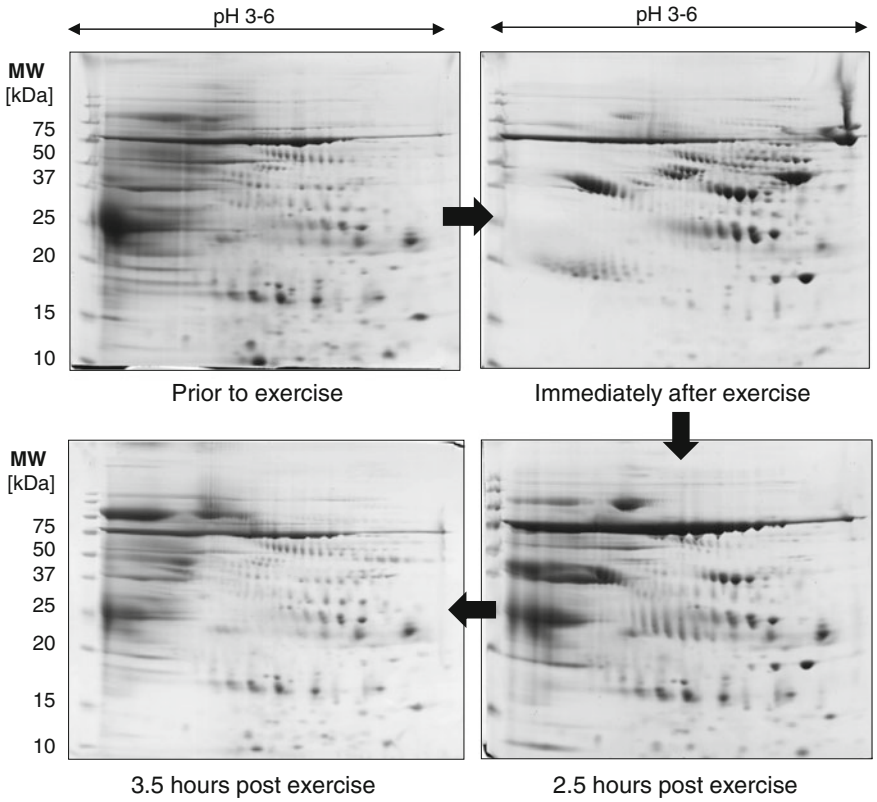

Fig. 12.1 Urinary proteome before and after exercise in 3,500 m altitude. **a** Prior to exercise, **b** first sample immediately post-exercise, **c** second sample after exercise (2.5 h post-exercise), and **d** third sample hours after exercise (3.5 h post-exercise)

was reported to have a higher impact on protein excretion than continuous cycling on a bicycle ergometer [21].

Running exercise at 70 % of the calculated maximal heart rate at normoxia condition and at hypoxia simulating 2,750, 3,250, and 3,750 m altitude showed that excreted protein amounts did not differ significantly. In contrast, specific proteins, namely albumin and β2-microglobulin, were significantly increased when training in hypoxia conditions [16] (Fig. 12.1).

12.5 Exercise and Acute or Chronic Kidney Injury

As exercise results in proteinuria, the question arises if exercise may lead to acute or chronic kidney injury. Investigation of urine samples from marathon runners showed that 40 % had elevated markers for acute kidney injury after the race. Acute kidney injury was determined by serum creatinine and urinary markers such as cystatin C, neutrophil gelatinase-associated lipocalin, and kidney injury molecule 1 supported the diagnosis. Cardiovascular magnetic resonance imaging results led to the suggestion that athletes were not volume-depleted, which could have triggered false conclusions. Eventually, all markers returned to normal baseline values after 24 h; however, repetitive long-distance running exercise has to be investigated concerning potential long-term alterations of kidney functions [19]. Urinary markers for renal damage were analyzed from patients with chronic kidney disease after a 20-min treadmill walk at 40–60 % exercise intensity. L-type fatty acid-binding protein (a new marker of tubular function in chronic kidney disease and acute kidney injury) as well as the common markers urinary albumin, N-acetyl-beta-D-glucosaminidase, and α1-microglobulin was not significantly influenced after exercise [11]. Junglee et al. [15] recently showed that muscle-damaging exercise prior to exercise in the heat can be seen as a risk factor for acute kidney disease.

Exercise of 15-min duration to maximal heart rate or exhaustion was shown to result in albuminuria. Within 24 h, measures returned to baseline in all subjects. It was therefore concluded that urinary parameters connected to disease or protein excretion should not be measured within 24 h after exercise [10].

12.6 Urinary Proteomics

In contrast to other body fluids, fewer studies were performed on the urinary proteome. Besides the fact that plasma or serum is the most common matrix in clinical studies, urine has a high salt concentration and is relatively dilute regarding proteins ($\sim$150 mg of protein/day) in general but still retains the issue of highly abundant albumin. On the other hand, urine can be sampled noninvasively and is available in large volumes of 1–2 l/day. Gel-based and gel-free proteomics was

used to map and categorize urinary proteins [2, 25, 34]. Adachi et al. [2] reported on an unexpectedly high percentage of membrane proteins. Compared to the entity of Gene Ontology entries, extracellular lysosomal and plasma membrane proteins were enriched and the latter were proposed to originate from renally eliminated exosomes. Such exosomes were subsequently investigated as potential source of new biomarkers [12].

12.7 Effect of Sports on the Urinary Proteome

Studies of untargeted analyses of urinary proteins or a certain fraction are rare. As summarized in the previous sections, exercise may change the urinary proteome differently depending on exercise intensity but also on temperature, hydration status as well as physiological condition. Besides parameters that result in variation of the proteome, Gür et al. described that the amount of protein excreted does not depend on age, duration of running or training, or athletic background of athletes as shown by examinations of participants of a half marathon [7]. The same exercise intensity may lead to different changes such as muscle damage or hematuria in some but not all athletes leading, e.g., to the excretion of myoglobin or hemoglobin and their fragments, which are usually not detected in urine. Therefore, even when measuring protein amounts and analyzing same amounts of proteins, substantial inter- and intra individual qualitative differences can occur. In addition to differences in renal filtration and reabsorption, proteins may not only originate from the kidney filtrate but could be post-renal, e.g., from parts of ureter, bladder, or urethra including proteins from the inner membranes as well as bacterial contamination through infection or other external contamination.

A pilot study using two-dimensional gel electrophoresis for comparison of the urinary proteome of elite athletes performing different types of exercise (endurance exercise, strength sport, and team sport) showed considerable differences within as well as between the groups [17]. This study was performed in a doping control context addressing the question how much the urinary protein varies and if that may influence the detection of, especially peptide and protein based, prohibited substances such as erythropoietin, insulin, or chorionic gonadotrophin. The idea was that in addition to the existing blood passport of athletes, a urinary passport or protein map may allow indication of, e.g., gene doping. In addition, from a sport physiological point of view, the urinary proteome could provide indications as to the nutritional and training status of an athlete. That way, muscle damage may be identified by myoglobin and its fragments in urine or erythrocytosis by the detection of hemoglobin or other erythrocyte-derived proteins. These parameters may be used for training control and modulation.

Protein maps of strength sport athletes, endurance sport athletes, and team sport athletes (10 each) showed that respective 2D patterns were too different within as well as between groups to allow a software-based comparison. Therefore, visual inspection was performed for evaluation of relevant differences. Proteinuria

(>15 mg protein/mmol creatinine) was found in 2/10 strength sport athletes, 5/10 team sport samples and 10/10 endurance sport samples and 0/10 samples from the control group. Endurance and team sport samples showed comparable protein patterns with protein spots considered as 'elevated' that contained transferrin, albumin, prostaglandin-H2 D-isomerase, immunoglobulin kappa chain and alpha-2-glycoprotein 1, gelsolin isoform b fragment, CD 201 antigen, kininogen 1, and clusterin isoform 1. In comparison, strength sport samples showed a higher amount of low molecular weight proteins (elevated spots contained transthyretin, CD 59 antigen, GM 2 ganglioside activator, and apolipoprotein A) including also fragments from high molecular weight proteins (albumin, transferrin, hemopexin, or IgG fragments) [17]. In this case, lactate cannot be the reason for increased protein excretion as the exercise time is too short for a sufficient production of lactate. Adrenergic activity is also discussed to be connected to proteinuria and results in higher blood pressure. As a matter of fact, blood pressure is extremely increased during weight lifting to values up to 370/360 mm Hg [22] and may be the reason for proteinuria in this case.

Within the same study, stability of urine samples was tested and it was found that the protein pattern did not change within four weeks of storage at 4 °C.

In a consecutive project, marathon runners who participated in the same marathon competition were investigated. As control groups, competitive athletes at rest with a mean endurance sport measure of 13–20 h/week (triathlon, biking, and running) were used. In addition, a control group of healthy volunteers performing occasional exercise (5 h/week) was acquired. No differences were found between the two control groups. Nine out of ten marathon runners had protein/creatinine ratios of >15 mg/mmol (15–73 mg/mmol). A relative decrease in acidic proteins was observed after exercise, which may be interesting for variations in EPO levels in doping control. Manual evaluation of gels showed six spots to be clearly elevated in marathon runners compared to healthy volunteers. Orosomucoid may be elevated because of increased glomerular filtration. If it can be shown that there is an increase in plasma as well, it could indicate an unspecific immune response due to muscle lesions. Another spot observed in marathon runners but not in controls contained hemopexin and may indicate hemolysis. The same phenomenon applies to a spot containing carbonic anhydrase I, which is responsible for the rehydration of carbon dioxide to bicarbonate within erythrocytes. Zinc α-2-glycoprotein 1, which is assumed to have a stimulatory effect on lipolysis, may be increased in plasma as well because of an increased energy consumption and demand. The increase of transferrin, an iron transport protein, may be due to glomerular filtration changes or to increased transferrin synthesis as athletes often have reduced iron levels [18]. For confirmation of the reason for higher concentration of these proteins in urine, plasma samples should be collected in addition in future studies.

In addition, it was investigated how the protein patterns change from rest to post-exercise and back in one volunteer in a yet unpublished study. The protein patterns changed after exercise and, within a few hours, returned back to the pattern observed prior to the intervention. Besides, it was found that high altitude (3,500 m, isobaric, 1 h) does not have an influence on the protein pattern or protein amount.

That is in contrast to residence in high altitude, where increased protein excretion is reported [32]. Exercise in altitude resulted in qualitatively similar protein patterns. Nevertheless, oxygen saturation was lower at high altitude, and power on a bicycle ergometer was lower.

Examples of urinary profiles of athletes from different sports categories are shown in Fig. 12.2 to illustrate the variability. Figure 12.3 shows different kinds of possible contaminations. The sample containing a high number of albumin fragments (encircled) is atypical, and the reason for the excretion of that many fragments in this individual case is unclear. The same is true for the gel which lacks albumin in usual amounts. For the sample containing myoglobin isoforms and fragments, the explanation may be that exercise has been muscle damaging. Nevertheless, there are several forms at different pI, which has not been described before.

In addition, other factors in athletes, such as special diets or training status, may lead to changes in metabolism and therefore also changes in the urinary protein pattern, which necessitates further investigations in future studies.

In summary, it can be concluded that it is critical to further investigate exercise-induced changes in protein patterns for different kinds of exercise conditions. Although the variations observed are very high, it is possible to find systematic

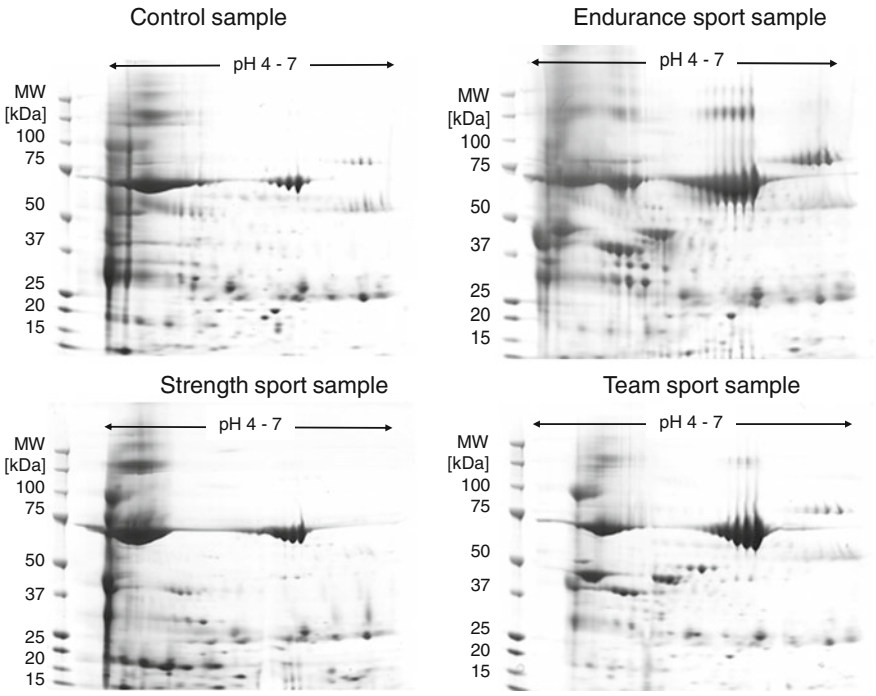

Fig. 12.2 Urinary protein pattern at rest and after endurance, team and strength sport

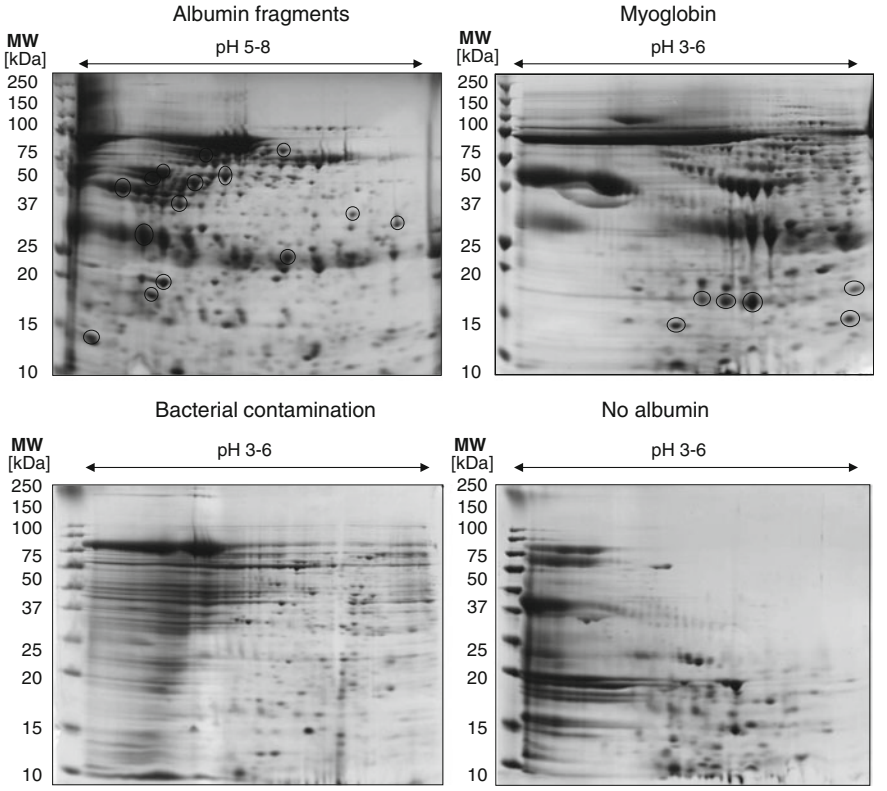

Fig. 12.3 Selected reasons for the variation of the urinary proteome and effect on the protein pattern. Samples with albumin fragments and myoglobin were from marathon runners after the race, and the samples with bacterial contamination as well as with the lack of albumin were from recreational endurance sport athletes after a time trial on a bicycle ergometer

changes for certain groups and it is important to expand the knowledge on exercise-induced changes for doping control, sport physiology as well as biomarker discovery in disease as levels of specific proteins may be changed after exercise.

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Chapter 13
Effects of Diuretics on Urinary Proteins

Xundou Li

Abstract Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood which has mechanisms to keep the internal environment homeostatic, urine is more likely to reflect changes of the body. As a result, urine is likely to be a better biomarker source than blood. However, since the urinary proteome is affected by many factors, including diuretics, careful evaluation of those effects is necessary if urinary proteomics is used for biomarker discovery. The human orthologs of most of these 14 proteins affected are stable in the healthy human urinary proteome, and 10 of them are reported as disease biomarkers. Thus, our results suggest that the effects of diuretics deserve more attention in future urinary protein biomarker studies. Moreover, the distinct effects of diuretics on the urinary proteome may provide clues to the mechanisms of diuretics.

Keywords Diuretics · Urine proteome

Biomarker is the measurable change associated with a physiological or pathophysiological process. Unlike blood is homeostatic, urine is more likely to reflect changes of the body. In other words, urine is likely to be a better biomarker source than blood [1]. Saving more urinary protein samples on the membrane can help to speed up the biomarker research in urine proteome [2]. Furthermore, compared to plasma, urine can be collected continuously and noninvasively. Second, the urinary proteome directly reflects the conditions of the urinary system. Third, it can also reflect the physiological status of the whole human body [3]. These advantages make the urinary proteome a suitable source for disease biomarker discovery.

To date, many urinary biomarkers have been reported in a variety of diseases [3], such as various chronic and acute renal injuries [4], bladder cancer [5], prostate cancer [6] and coronary artery disease [7]. However, studies focusing on the urinary

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protein biomarker discovery still face certain challenges. A major issue is that the urinary proteomic pattern of an individual may be affected by multiple factors, such as gender, age, diet [8], medication, daily activities, exercises [9, 10], smoking [11], stress, menstrual cycle, and other physiological variations. Environmental factors including temperature and humidity may also affect the urinary proteome. Therefore, these factors should be taken into consideration in the urinary biomarker research.

Effects of some factors, such as gender, age, daily activity, and environmental conditions, have been investigated previously [12–14]. However, effects of some other factors, especially medication, are difficult to examine, since the regular therapeutic process of patients should not be disturbed during the collection of urine. Therefore, influences of medications on the urinary proteome should be taken into account during data analysis and interpretation.

Diuretics are among the most commonly used medications. They are used to induce negative fluid and sodium balances in a variety of clinical situations, including hypertension, heart failure, renal failure, nephritic syndrome, and cirrhosis [15]. However, it remains unclear whether and how diuretics affect the urinary proteome, which hampers the urinary biomarker discovery for those diseases.

The effects of furosemide, hydrochlorothiazide, and spirolactone on the urinary proteome were examined using label-free quantitative proteomics [16]. These drugs represent thiazide diuretics, loop diuretics, and potassium-sparing diuretics, respectively, which are the three types of commonly used diuretics with different modes of action [17]. The rat urine samples were collected before and after the diuretics were administered, digested using the filter aided proteome preparation (FASP) method [18], and analyzed with a high-speed TripleTOF 5600 system. Progenesis LC-MS was used to quantify the urinary proteins.

Urine samples from 15 rats were collected after each rat was given 1 ml saline by intragastric administration for 24 h using metabolic cages, and these samples were used as controls. Then, the rats were randomly divided into three groups with five rats in each group. Each group of rats was given either 20 mg/(kg days) of furosemide, 20 mg/(kg days) of spirolactone, or 25 mg/(kg days) of hydrochlorothiazide, respectively. The dosing volumes of diuretics were adjusted to 1 ml. All rats were given diuretics by intragastric administration for 5 days, and the rat urine samples were collected on 1, 3, and 5 days after diuretics administration as described above.

As shown in Table 13.1, the rat urinary volumes increased significantly ($\sim$2–3, $P < 0.05$) after the administration of furosemide (F) and hydrochlorothiazide (H), especially within the first 8 h after lavage. This period is the effective time of the diuretics. However, there is no significant increase in urine output ($P > 0.05$) after the rats were administered with spirolactone (S), probably due to the fact that spirolactone is not an efficient diuretic on its own and usually is applied in combination with other diuretics.

As a first step of the sample analysis, the urine samples collected on different days were separated by SDS-PAGE. As shown in Fig. 13.1a, the protein patterns of

Table 13.1 The urine volumes of rats before and after diuretics were administered [16]

Time	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Mean; SD (8 h)
	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	
Furosemide											
Before	3.6	37.7	2.8	11.4	3.7	10.0	3.0	11.5	3.3	8.5	3.2; 0.3
Day 1*	7.7	38.7	3.6	15.6	9.2	22.2	6.5	27.5	5.0	13.0	6.4; 2.2
Day 3*	7.2	42.2	2.5	10.0	8.0	20.6	6.2	30.0	7.2	14.5	6.2; 2.2
Day 5*	10.0	21.5	8.0	13.5	9.0	14.0	13.3	21.3	5.5	8.5	9.2; 2.8
Hydrochlorothiazide											
Before	5.5	12.5	6.1	30.0	5.3	11.0	4.9	15.5	4.7	12.5	5.3; 0.5
Day 1*	12.0	27.5	13.4	34.9	12.0	28.2	9.5	19.7	10.5	23.0	11.5; 1.5
Day 3*	9.3	19.3	12.0	38.0	11.3	25.0	5.3	15.3	15.5	23.5	10.7; 3.7
Day 5*	16.0	27.0	12.5	21.0	10.5	20.5	11.0	17.0	12.0	18.0	12.4; 2.2

(continued)

Table 13.1 (continued)

Time	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5		Mean; SD (8 h)
	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	Urine volume (8 h) ml	Urine volume (24 h) ml	
Spirolactone											
Before	–	27.5	–	15.5	–	27.5	–	17.5	–	14.2	–
Day 1**	–	36.0	–	18.5	–	28.0	–	18.0	–	16.0	–
Day 3**	–	23.0	–	15.5	–	27.0	–	17.4	–	21.0	–
Day 5**	–	25.0	–	17.0	–	29.5	–	19.0	–	20.5	–

Note *P values <0.05; **P values >0.05 (T-test)

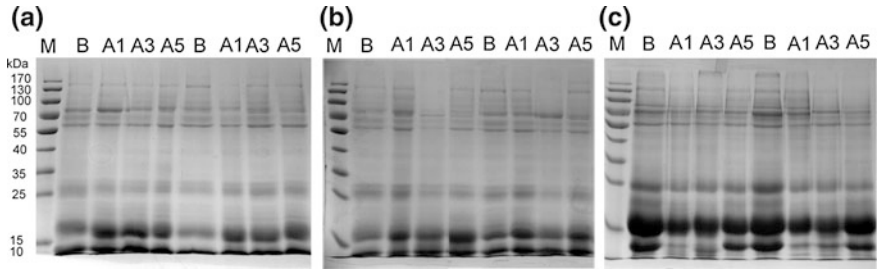

Fig. 13.1 SDS-PAGE of the urine samples from rats treated by different diuretics [16]. Urine protein samples were separated by SDS-PAGE and stained using Commassie *blue* brilliant for the hydrochlorothiazide group (H, **a**), the furosemide group (F, **b**), and the spirolactone group (S, **c**), *M* markers; *B* normal rat urine samples; *A1*, *A3,* and *A5* urine samples obtained 1, 3, and 5 days after the diuretics were administered

the urine samples in the H group changed only modestly among those obtained before and 1, 3, and 5 days after the diuretics administration. However, for the F and S groups, there were some significant changes among samples obtained at different time points, especially those on Day 3 after gavage for the F group (Fig. 13.1b) and Day 1 for the S group (Fig. 13.1c). Therefore, normal urine samples, Day 3 for the F and H groups and Day 1 for the S group, were further analyzed using 1D-LC-MS/MS.

To investigate the changes of the urine proteome after diuretics administration, a total of 18 LC-MS/MS runs of urine samples from three different rats in each diuretic group were analyzed. The 18 datasets were analyzed using Progenesis LC-MS, and Mascot Daemon. The false discovery rate (FDR) was adjusted to be less than 1 %. As a result, there were 331, 302, and 325 proteins identified in the F, S, and H group, respectively.

The coefficients of variation (CVs) for each of the three levels of sample variation before gavage, after gavage, and between these two conditions were calculated. As shown in Fig. 13.2, the CV values of the samples after gavage were slightly higher than those before gavage (median CV values: F group 0.25 vs. 0.34; S group 0.35 vs. 0.39; H group 0.28 vs. 0.31), possibly maybe because rats respond differentially to the diuretics. In contrast, the CV values of the samples for between before and after gavage and for after gavage (median CV of F group is 0.45; median CV of S group is 0.55) are significantly higher ($P < 0.05$), suggesting that furosemide and spirolactone can change the urine proteome. However, the CV values of H-diuretics (median CV is 0.33) were not changed significantly, indicating that hydrochlorothiazide has no discernible effects on the rat urine proteome at this dosage.

The CV values of proteins identified in each group before diuretics administration, after and between these two states, were calculated using SPSS 13.0. Before indicates the CV values of urine samples before diuretics administration in the F, S, and H group, respectively; after indicates the CV values of urine samples after diuretics administration in each group; between indicates the CV values of urine

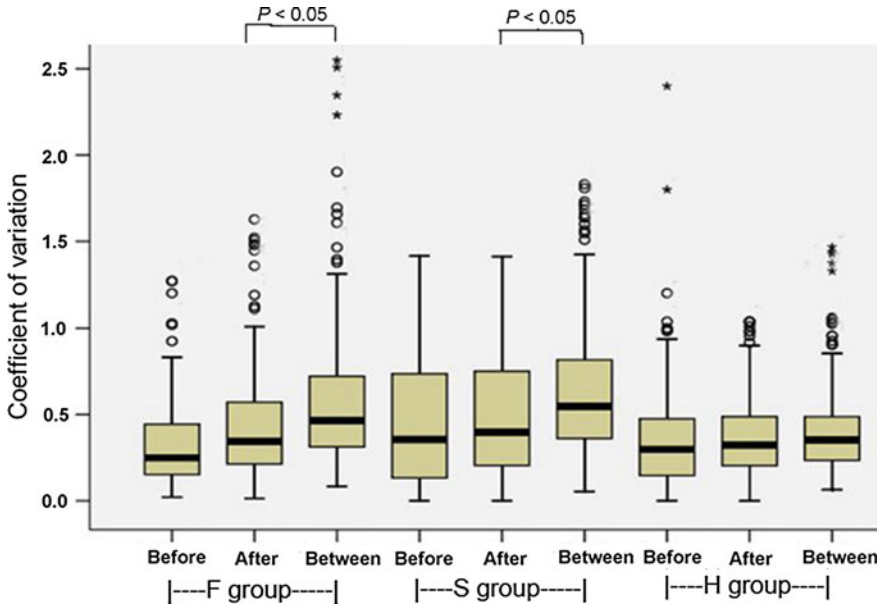

Fig. 13.2 The CV values for each of the three levels of sample variation [16]

samples between before and after diuretics administration in each group ($n = 3$; in F and S group, $P < 0.05$).

Using the label-free quantification by the Progenesis LC-MS software, we identified seven (five upregulated and two downregulated), five (one upregulated and four downregulated), and two (one upregulated and one downregulated) proteins which significantly changed in all three rats in the F, S, and H group, respectively, according to the criteria: $P \leq 0.05$, a fold change ≥2 and a spectral count ≥5. As shown in Tables 13.2 and 13.3, five of the seven proteins in the F group and all of the five proteins in the S group have been reported to be disease biomarkers. For example, haptoglobin is a candidate biomarker for patients with bladder cancers, acute kidney injury, or diabetic nephropathy. However, neither of the two significantly changed proteins (beta-microseminoprotein and EGF-containing fibulin-like extracellular matrix protein 1) has been reported as biomarkers in hydrochlorothiazide group. Moreover, hydrochlorothiazide appears to have a lower impact than furosemide and spirolactone at the dosages tested. Interestingly, no significantly changed proteins are shared by any two groups, indicating the distinct effects of the diuretics on the urinary proteome.

We next evaluated the relevance of our findings to the human disease biomarkers. As it is typically assumed that orthologs (co-orthologs) retain similar functions between species [19, 20], therefore we transformed the significantly changed proteins after intragastric administration of diuretics to human orthologs. Based on the 122.R_norvegicus.orthologues database and Ensembl Compare

Table 13.2 Urinary proteins significantly changed after intragastric administration of furosemide [16]

Accession	Protein name	Fold change			Candidate biomarkers	References
		Rat 1	Rat 2	Rat 3		
P02781	Prostatic steroid-binding protein C2	8.2↑	6.3↑	4.3↑	No	
P07647	Submandibular glandular kallikrein-9	3.5↑	6.2↑	5.2↑	Yes	[24]
P02782	Prostatic steroid-binding protein C1	7.6↑	5.7↑	5.6↑	No	
P02780	Secretoglobin family 2A member 2	9.6↑	5.0↑	6.2↑	Yes	[24]
P22283	Cystatin-related protein 2	4.7↑	3.7↑	4.3↑	Yes	[24]
P08721	Osteopontin	7.3↓	7.4↓	5.9↓	Yes	[25–28]
Q01177	Plasminogen	2.1↓	2.1↓	3.0↓	Yes	[29]

Table 13.3 Urinary proteins significantly changed after intragastric administration of spirolactone [16]

Accession	Protein name	Fold change			Candidate biomarkers	References
		Rat 1	Rat 2	Rat 3		
P06866	Haptoglobin	5.0↑	2.1↑	2.2↑	Yes	[30–35]
P81828	Urinary protein 2	3.6↓	3.3↓	3.9↓	Yes	[24]
P81827	Urinary protein 1	7.3↓	4.3↓	4.4↓	Yes	[24, 36]
P10960	Sulfated glycoprotein 1	4.0↓	3.1↓	2.4↓	Yes	[24]
Q09030	Trefoil factor 2	8.5↓	4.7↓	4.2↓	Yes	[37]

database [21], eight of the 14 rat urinary proteins have human orthologs (Table 13.4). By comparing the proteins with the human core urinary proteome, we further found that seven human orthologs are relatively stable proteins in the normal human urinary proteome [22, 23]. Therefore, such proteins could serve as potential urinary biomarkers, since significant qualitative or quantitative changes of these stable proteins may suggest some pathophysiological conditions [23].

However, some limitations of this study should be noted. First, the results need to be verified on humans before we can generalize the conclusions. Second, it would be ideal to validate our results in studies with a higher statistical power. Furthermore, the effects of doses and durations of diuretics on the urinary proteome should also be studied in the future.

In summary, we have shown for the first time through a proteomic approach that some candidate urinary biomarkers may be affected by diuretics, suggesting that the

Table 13.4 Human orthologs of rat proteins significantly changed after administration of diuretics [16]

Rat protein ID	Rat protein name	Human protein ID	Human protein name	Human core urinary proteome
Q01177	Plasminogen	P00747[a]	Plasminogen	Yes
Q09030	Trefoil factor 2	Q03403[a]	Trefoil factor 2	Yes
P08721	Osteopontin	P10451[a]	Osteopontin	Yes
O35568	EGF-containing fibulin-like extracellular matrix protein 1	Q12805[a]	EGF-containing fibulin-like extracellular matrix protein 1	Yes
P10960	Sulfated glycoprotein 1	P07602[a]	Sulfated glycoprotein 1	No
P06866	Haptoglobin	P00738[a]	Haptoglobin	Yes
P02781	Prostatic steroid-binding protein C2	P11684[b]	Secretoglobin family 1A member 1	Yes
P07647	Submandibular glandular kallikrein-9	P06870[b]	Kallikrein-1	Yes

Note [a] Present in the 122.R_norvegicus.orthologues database
[b] Present in the Ensembl Compare database

effects of diuretics should be carefully evaluated in the future urinary protein biomarker studies. The results obtained here could help minimize the interference of diuretics with biomarker discovery using the urinary proteomics. In addition, the significantly changed proteins may help the investigation of mechanisms of diuretics as well as renal clearance of proteins. Other commonly used medications, such as glucocorticoids and angiotensin-converting enzyme inhibitors (ACEIs), may likewise affect the urinary proteome and should also be further studied.

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Part IV
Discovery of Kidney Disease Biomarkers

Chapter 14
Applications of Urinary Proteomics in Renal Disease Research Using Animal Models

Yang Lv, Guangyan Cai and Xiangmei Chen

Abstract Animal models of renal disease are essential tools in research on kidney disease and have provided valuable insights into pathogenesis. Use of animal models minimises inter-individual differences, allows specific pathological changes to be examined, and facilitates collection of tissue samples. Thus, mechanistic research and identification of biomarkers are possible. Various animal models manifesting specific pathological lesions can be used to investigate acute or chronic kidney disease (CKD). Urine, a terminal metabolic product, is produced via glomerular filtration, reabsorption, and excretion in the tubular and collecting ducts, reflecting the functions of glomeruli or tubular tissue stimulated in various ways or subject to disease. Almost 70 % of urinary proteins originate from the kidney (the other 30 % come from plasma), and urinary sampling is important to noninvasively detect renal disease. Proteomics is powerful when used to screen urine components. Increasingly, urine proteomics is used to explore the pathogenesis of kidney disease in animals and to identify novel biomarkers of renal disease. In this section, we will introduce the field of urinary proteomics as applied in different models of animal renal disease and the valuable role played by proteomics in noninvasive diagnosis and rational treatment of human renal disease.

Keywords Urine proteomics · Renal diseases · Animal models

14.1 Progress in Research on Animal Models of Renal Disease

In terms of pathological features, three types of animal model of renal disease may be identified. These are models of acute glomerulonephritis, acute renal tubular injury, and chronic kidney disease (CKD). The principal injury in models of acute

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glomerulonephritis is to the glomeruli; models of acute renal tubular injury exhibit lesions principally of the renal tubules and interstitia; CKD models feature pathological changes in both the glomeruli and tubules.

There are three principal types of acute glomerulonephritis models, the mesangial proliferative glomerulonephritis model, the membranous nephropathy model, and the anti-glomerular basement membrane nephritis model. Proliferative mesangial models, including the rat acute anti-Thy1 nephritis model and mouse snake venom-triggered glomerulonephritis, are characterised by initial mesangiolysis, followed by mesangial cell proliferation and accumulation of mesangial matrix, in turn causing proteinuria. The conditions subsequently resolve, and the histology becomes almost normal [9, 12]. Membranous nephropathy models, such as the passive Heymann nephritis model (PHN), are characterised by the formation of membrane attack complexes inducing injury to podocytes and expansion of the glomerular basement membrane, in turn causing high-level proteinuria [10, 27]. The anti-glomerular basement membrane nephritis model exhibits proliferative glomerulonephritis accompanied by crescent formation and proteinuria and is triggered by the injection of purified anti-GBM immunoglobulin into the tail vein [4, 5, 17]. All of these models find applications in studies of the pathogenesis of glomerular disease and in attempts to identify useful interventions.

Acute renal tubular injury models include the unilateral ureteral obstruction (UUO) model, the ischaemia–reperfusion model, models of drug-induced disease, and crush syndrome (CS) models. The UUO model, featuring unilateral urethral ligation, may trigger renal tubular atrophy and early interstitial fibrosis, but glomerular function remains normal. UUO has been used to induce tubulointerstitial damage and interstitial fibrosis and is important in studies of the mechanisms of renal fibrosis and evaluation of various therapeutic approaches [7, 8]. The rat ischaemia–reperfusion model features renal pedicle occlusion for 45 min, followed by the restoration of blood supply, and is used to study the pathogenesis and mechanisms of acute renal tubule damage [11]. Drug-induced models of renal injury feature obvious injuries to the tubules and interstitia. Nephropathy induced by aristolochic acids and cyclosporine is characterised by the development of tubular lesions, interstitial fibrosis, hyaline degeneration, and damage to small arteries of the kidney [2]. Gentamicin and cisplatin induce acute renal failure characterised by tubular injury and renal tubular epithelial cell death; both apoptosis and necrosis may be in play [6, 21]. Research using such models aids in understanding of the pathogenesis of drug-induced kidney injuries and can be used to develop new approaches to treatment. CS models of acute kidney injury (AKI) simulate the major shock and renal failure developing after crushing of skeletal muscle. The pathogenesis includes rhabdomyolysis, which may be induced by either compression or injection. Akimau et al. [1] and Murata et al. [15] compressed rat hind limbs with weights and found lesions in epithelial cells and the distal tubules, but the glomeruli and proximal tubules were normal. Blachar et al. [3] developed a CS model by infusing 100 mg of muscle protein/kg body weight intravenously in rabbits; renal lesions (including vacuolation of tubular cells and

formation of homogeneous eosinophilic casts) developed. The CS model of Wang et al. [26] features renal tubular cell apoptosis triggered by intramuscular injection of 8 ml/kg body weight of glycerol. In this model, injection of fasudil ameliorated tubular injury by suppressing apoptosis. CS models are used to explore the pathogenesis of AKI induced by rhabdomyolysis and to develop effective therapeutic approaches.

The principal CKD models include the nephrectomy model and the chronic anti-Thy1 nephritis model. Lesions develop in the glomeruli and tubules. In the commonly used 5/6 nephrectomy model, the remnant kidney usually exhibits early compensatory hypertrophy, glomerular perfusion, a high filtration rate, and hyperpressure; these developments are followed by glomerular sclerosis, capillary loop collapse, progressive mesangial expansion, tubular interstitial damage, and, ultimately, progressive renal failure [22]. Also, rats from which two-thirds of kidney tissue has been removed have been used to investigate residual renal destruction [24]. However, this model is not widely employed. Chronic anti-Thy1 nephritis, triggered by a single injection of anti-Thy1 antibody into unilaterally nephrectomised rats, causes development of glomerular necrosis, renal fibrosis, and a decline in renal function 1 month after injection [25]. The various CKD models are used to explore the pathogenesis of renal insufficiency and renal failure.

14.2 Urine Proteomics of Normal Animals

The protein profiles of normal urine have been described. Thongboonkerd et al. [23] analysed urine proteins of SD mice using 2D-PAGE, followed by MALDI-TOF-mediated protein identification. A total of 350 protein spots were found and 111 identified. The proteins included transporters, transport regulators, enzymes, signalling proteins, cytoskeletal proteins, signal-binding proteins, and receptors. The cited authors also [23] investigated the urine proteins of rats acutely overloaded with sodium. The levels of all of neutral endopeptidase, proteins of solute carrier family 3, meprin 1α, diphor-1, heat-shock protein 72, vacuolar H^+ATPase, ezrin, ezrin/radixin/moesin-binding protein, glutamine synthetase, and guanine nucleotide-binding protein fell, whereas those of albumin and α-2u globulin rose, compared to controls. Such changes were suggested to be associated with tubular transport of sodium.

14.3 Urinary Proteomics in Renal Disease Animal Models

Currently, research focus is on the use of the renal AKI model (featuring tubular lesions). For example, Rucevic et al. [20] used proteomics to explore the effects of 4 days of aristolochic acid treatment on the urine proteins of DBA and C57BL mice. The levels of various proteins, including those of the cytoskeleton and those

involved in kidney development and inflammation, changed in response to induced renal injury. The results aid the diagnosis and treatment of human aristolochic acid nephropathy. Rouse et al. [18] examined urinary proteins in a model of gentamicin-induced kidney disease. The levels of collagen type I and III fragments were elevated, in agreement with histopathological data. Also, m- and a-glutathione S-transferases (mGst and aGst), renal papillary antigen-1 (Rpa-1), kidney injury molecule-1 (Kim-1), lipocalin-2 (Lcn-2), osteopontin (Opn), and clusterin (Clu) were present at notably higher levels in urine 1–3 days after gentamicin treatment, but the levels of mGst, aGst, and Rpa-1 recovered to normality at day 10 and those of Kim-1, Lcn-2, Clu, and Opn recovered at day 15 [19]. More importantly, the level of Rpa-1 reflected repair and recovery of the tubular and collecting ducts, and Rpa-1 may serve as a biomarker of tubular regeneration. Zhou et al. [29] examined urine proteins in a cisplatin-induced AKI model. Urinary exosomes isolated by differential centrifugation were analysed by 2D differential gel electrophoresis and the proteins identified using MALDI-TOF-TOF or LC-MS/MS. A total of 18 proteins were upregulated and 9 downregulated 8 h after cisplatin injection. The fetuin-A level increased 52.5-fold by day 2 (1 day before the rise in serum creatinine) and remained elevated to day 5 (the peak of renal injury) after cisplatin injection. The urinary fetuin-A level increased 31.6-fold in the early phase (2–8 h) of reperfusion after ischaemia and was elevated in three ICU patients with AKI compared to those without AKI. Thus, the urinary fetuin-A level may serve as a biomarker for early diagnosis of AKI and may predict the extent of renal injury. Maddens et al. [13] induced AKI by the inoculation of *Escherichia coli* into aged mice in which uterine ligation had been performed. Urinary chitinase-3-like proteins 1 and 3 were detected only in septic mice with severe AKI. Also, the human homologue, chitinase 3-like protein 1, was present at higher levels in urine of septic patients with AKI than without, supporting the notion that urinary chitinase 3-like protein 1 may play a role in human infection-induced AKI. Such work may identify biomarkers reflecting the level of tubule damage, and this will aid in the noninvasive diagnosis of AKI patients.

Proteomics has been used in the context of other models of renal disease. Wu et al. [28] compared urine proteins in mice with moderate and severe immune-mediated nephritis triggered by the injection of anti-GBM antibody and found that severely injured mice expressed significantly higher urinary levels of vascular cell adhesion molecule-1 (VCAM-1), P-selectin, tumour necrosis factor receptor I (TNFRI), and CXCL16, suggesting that these proteins were associated with the development of spontaneous immune nephritis. Nabity et al. [16] analysed the urine proteins of dogs with X-linked hereditary nephropathy (XLHN). Retinol-conjugated protein (RBP) was first detected in urine approximately 2 months before azotaemia development, and the RBP level increased as disease progressed, suggesting that urinary RBP might serve as a biomarker for the early detection of tubulointerstitial damage. Moreno et al. [14] used SELDI-TOF to define serum and urinary biomarker signatures associated with a rapid therapeutic response to a cyclin-dependent

kinase (CDK) inhibitor in the jck mouse model of PKD and found that 20 urinary and 21 serum biomarkers might aid further assessment of CDK inhibitors as therapeutic agents for the condition.

14.4 Conclusion

Urinary proteomics will undoubtedly contribute to future research on kidney disease. Many studies have shown that urinary proteomics aids in the understanding of pathophysiological mechanisms and the discovery of novel biomarkers and therapeutic targets. However, the scope of urine proteomics is limited, and some commonly used renal disease models, including the glomerulonephritis and CKD model, are not currently amenable to further analysis using urine proteomics. Therefore, future developments in urine proteomics are required for detailed investigation of the molecular mechanisms of various animal disease models and identification of novel biomarkers, eventually guiding noninvasive diagnosis of and effective therapy for human renal diseases.

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Chapter 15
The Application of Urinary Proteomics for the Detection of Biomarkers of Kidney Diseases

Song Jiang, Yu Wang and Zhihong Liu

Abstract Urine is a biological material that can be easily obtained in the clinic. The identification of proteins excreted in urine provides useful biological information about the kidney as well as a unique opportunity to examine physiological and pathological changes in the kidney in a noninvasive manner. Recent technological advances in urinary proteomic profiling have provided the foundation for a number of urinary proteomic studies directed at identifying markers of kidney disease diagnosis, prognosis, or responsiveness to therapy. In this review, we describe the strengths of different urinary proteomic methods for the discovery of potential biomarkers of kidney diseases. We also highlight the limitations and future goals of these approaches.

Keywords Urinary proteomics · Biomarker · Kidney diseases

15.1 The Urinary Protein Profile

Under normal physiological conditions, a person's daily urine output contains <20 mg of albumin and <150 mg of total protein. Approximately 30 % of this protein content is derived from plasma, whereas 70 % is produced by the kidney and the lower urinary tract [32]. Normal urine contains at least 1,500 proteins, most of which are extracellular and membrane bound [1]. To be present in the urine, proteins or their fragments must pass through filters at the glomerulus and bypass or otherwise avoid tubular reabsorption. Alternatively, proteins can be secreted by the kidney or lower urinary tract directly into urine. During plasma filtration in the glomeruli, the glomerular capillary walls discriminate among molecules of different sizes, charges, and configurations. The glomerular basement membrane and the slit diaphragm of the filtration barrier limit the passage of macromolecules containing negatively charged glycosaminoglycans. Small, positively charged molecules could be filtrated

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in different ways. Typically, proteins <20 kDa can move freely, whereas proteins >60 kDa are almost completely restricted in their movement between compartments. Despite this filtering, the most abundant urine protein is albumin, a negatively charged molecule with a molecular weight of approximately 66.4 kDa. The relative abundance of albumin in the urine may be due to the presence of large pores through which albumin, immunoglobulins, and other macromolecules can pass [11].

Injury to any of the filtration barrier structures results in the leakage of large, negatively charged proteins, thereby increasing the presence of these proteins in urine; for example, in diabetic nephropathy (DN) and focal and segmental glomerulosclerosis (FSGS) [39], tubules reabsorb most of the filtered proteins. Proximal tubules also catabolize proteins and excrete the resulting peptides into the urine. Tubules secrete proteins directly into urine during normal maintenance processes or in response to injury. Tubular injury may result in the decreased reabsorption or catabolism of the filtered proteins and in the secretion of tubular proteins in response to the injury. In addition to soluble proteins and their peptides, urine also contains exosomes, which are specialized vesicles that are shed by the renal epithelia directly into the urine [21, 33]. The distal organs of the lower urinary tract also contribute to the urinary proteome.

15.2 Urinary Proteomics Approaches

Typically, proteomic biomarker studies consist of 2 main stages: a discovery phase and a validation phase. The discovery phase can be divided into 3 main steps: (1) sample preparation, (2) mass spectrometry analysis, and (3) data analysis. In the discovery phase, a variety of proteomic methods have been used to identify biomarkers of kidney disease, including liquid chromatography mass spectrometry (LC-MS), two-dimensional electrophoresis mass spectrometry (2DE-MS), surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS), and capillary electrophoresis combined mass spectrometry (CE-MS) [14, 23, 34, 36]. Traditional biochemical methods such as enzyme-linked immunosorbent assays (ELISAs) and Western blotting (WB) are widely used in the validation stage.

15.3 The Study of Kidney Disease Through Urinary Proteomics

15.3.1 Diabetic Nephropathy

DN is a complication of diabetes that affects up to 40 % of patients with diabetes. In the Western world, DN is the leading cause of end-stage renal disease (ESRD) [37]. Given the increasing incidence of diabetes [50], DN has already become a major cause of chronic kidney disease in China.

Microalbuminuria (MA) was wildly used as an early diagnostic marker of DN. However, long-term longitudinal studies have shown that only a subset of patients with MA progress to proteinuria [13, 28, 29]; indeed, many individuals with Type 1 diabetes have already experienced early renal function decline before or coincidental with the onset of MA [30, 31]. These data suggest that MA may be an inadequate early diagnostic biomarker of DN, spurring an intense search for new biomarkers of DN using proteomic techniques.

To identify more sensitive and specific biomarkers of DN Rossing et al. [38], designed a proteomic panel capable of distinguishing diabetes from DN in 305 individuals using CE-MS. The sensitivity and specificity of this panel was 97 %. Further study has shown that this panel has predictive value for the progression of MA toward overt DN over a 3-year follow-up period. To validate this result, a multicenter study involving 145 patients with Type 2 diabetes was initiated [2]. In this independent cohort, the diagnostic panel for DN displayed 93.8 % sensitivity and 91.4 % specificity, with an AUC of 0.948. Statistical analysis demonstrated that the DN diagnostic model score was well correlated with clinical parameters such as presence of albuminuria and the estimated glomerular filtration rate (GFR).

To further explore the underlying pathogenesis of renal function decline in DN with MA proteinuria and to identify a discriminating biomarker, Merchant et al. [23] used LC-MALDI-TOF-MS to analyze the urinary peptidome of a long-term longitudinal DN cohort with MA. In the urine of patients with early renal function decline, 3 peptides with decreased content and 3 peptides with increased content were identified. Of the 3 peptides with increased content, high levels of 2 were observed in renal biopsy tissue from Type I diabetes patients suffering from early nephropathy. This result indicates that these peptides have potential for use as early diagnostic biomarkers, although their sensitivity and specificity remain to be validated in clinical practice.

ITRAQ-labeled LC-MS has recently become a popular proteomic technology. This method was used to identify urinary proteomic biomarkers that may enable the diagnosis of DN in a group of Type 2 diabetes patients with or without MA [18]. Some differentially excreted proteins were verified by multi-reaction mass spectrometry (MRM) analysis of urine collected from 9 individual normoalbuminuric and 14 individual microalbuminuric patients. α-1-Antitrypsin, α-1-acid glycoprotein 1, and prostate stem cell antigen all yielded excellent AUC values (0.849, 0.873, and 0.825, respectively).

15.3.2 IgA Nephropathy

IgA nephropathy (IgAN) is the most common glomerular disease worldwide. The prevalence of this disease is highest in Asian populations, intermediate in European populations, and lowest in African populations. The clinical presentation of IgAN is variable and includes isolated hematuria, rapidly progressive loss of renal function, or full nephrotic syndrome. Similarly, the histological features of IgAN range from

mesangial proliferation to glomerular extracapillary proliferation with crescent formation. In current clinical practice, the clinical and morphological features of IgAN are inadequate to precisely classify its molecular mechanisms or predict the disease outcome or responsiveness to therapeutic intervention.

Several studies have examined the urinary proteome to explore novel biomarkers. He's group identified a panel of 10 urinary proteins (of which 8 were upregulated and 2 were downregulated); the expression of which differed in patients with IgAN and healthy individuals. Moreover, this panel distinguished patients with severe IgAN from those with mild IgAN with 90.48 % sensitivity and 96.77 % specificity [15]. Brigitte et al. analyzed the urinary proteomes of patients with IgAN and healthy individuals using 2 DE-MS and demonstrated that the laminin G-like 3 (LG3) fragment of endorepellin was decreased in the IgAN group [41]. This finding was subsequently validated in 43 IgAN patients and their corresponding controls by ELISA. Statistical analysis indicated a significant inverse correlation between LG3 levels and the glomerular filtration rate of IgAN that was not observed in 65 patients with other glomerular diseases. These results suggest that the LG3 fragment of endorepellin is a potential biomarker of IgAN severity.

Distinct urinary protein profiles distinguishing healthy individuals and patients with IgAN have also been identified [27, 52], although these findings have not been applied in a clinical setting to confirm the clinical utility of urinary protein profiling.

Urinary proteomic methods have also yielded potential predictive markers of the response of IgAN to intervention. For example, the urinary proteomic profile of patients with IgAN predicted their response to angiotensin-converting enzyme (ACE) inhibitors and urine levels of kininogen-1, inter-α-trypsin inhibitor heavy chain H4, and transthyretin differed significantly between ACE inhibitor therapy responders and nonresponders [36]. Very low urinary levels of kininogen-1 were correlated with a poor response to this treatment. Studies with large sample sizes will be needed to evaluate the clinical applicability of these urinary protein markers.

15.3.3 Membranous Nephropathy

Membranous nephropathy (MN) is a common type of primary glomerulonephritis in North America, Europe, and Asia. In severe cases, MN can produce ESRD [22]. This antibody-mediated autoimmune glomerular disease is characterized by the presence of immune deposits on the epithelial side of the glomerular capillary wall. Our understanding of the pathogenesis of membranous nephropathy is mostly derived from studies in rats with passive Heymann nephritis (PHN), a glomerular disease that closely resembles human membranous nephropathy. In rats, PHN can be induced by a single injection of heterologous antiserum or IgG against renal tubular cell antigens [19].

The discovery of the anti-phospholipase A2 receptor (PLA2R) antibody greatly improved our understanding of the molecular mechanisms of MN. Serum levels of the anti-PLA2R antibody are used to guide diagnosis, monitor disease activity, and

assess the response to treatment in patients with membranous nephropathy [5, 6, 35]. However, PLA2R is inadequate for the management of these patients, and nephrologists are seeking to identify additional biomarkers with clinical utility [8].

A serial analysis of the urinary proteomic profile of rats based on urine samples collected at days 0, 10, 20, 30, 40, and 50 after PHN induction [26] revealed that 37 proteins were differentially expressed across these time points. The differentially expressed proteins were classified into several categories: proteins that decreased after PHN induction; proteins that increased after PHN induction; proteins that increased during the early phases of PHN but returned to basal levels in later phases; proteins that were undetectable during PHN; and proteins that were detectable only during PHN. Most of the differentially expressed proteins are related to signaling pathways, protein trafficking, and the regulation of glomerular permeability.

Urinary proteomics studies addressing MN are rare due to limitations in the technology used to detect protein profiles in mass proteinuria. However, kidney and podocyte proteomic studies of human MN are ongoing [47]. Comparative studies of kidney or podocyte proteomes and urinary proteomes will likely represent a breakthrough in this field.

15.3.4 Focal Segmental Glomeruloscelerosis

Focal segmental glomeruloscelerosis (FSGS) is a major cause of proteinuria and renal failure [20]. This disease comprises a number of clinical and pathological syndromes that share a common glomerular lesion, including primary (or idiopathic) FSGS, secondary FSGS (mediated by glomerular hypertension and hyperfiltration), and genetic, virus-associated, and drug-induced forms of the disease [10]. Histologically, FSGS is classified into several subtypes, including tip variant, perihilar variant, cellular variant, collapsing variant, and "not otherwise specified" FSGS [9, 43].

The critical clinical feature of FSGS is proteinuria. To distinguish FSGS-induced proteinuria from other proteinuria diseases based on proteins present in the urine, Sanju et al. [44] used 2-DE to compare urine samples from 32 patients with proteinuria-causing diseases including FSGS, lupus nephritis (LN), MN, and DN. Differentiated spots from 16 patients were used to train an artificial neural network to create a prediction model, which then was validated in the remaining 16 patients. The model achieved sensitivities of between 75 and 86 % and specificities of between 67 and 92 %.

Glucocorticoids are the main intervention for FSGS; however, not all patients respond to glucocorticoid treatment. Nuntawan et al. compared the urinary proteomic profile of steroid-resistant nephrotic syndrome (SRNS) with that of steroid-sensitive nephrotic syndrome (SSNS) using SELDI-TOF-MS [34]. A 13.8-kDa-fragment of α-1-β glycoprotein was significantly differentially excreted between these 2 groups. The results of the validation study demonstrated that this peptide was present in 7 of

the 19 SRNS patients but absent in all SSNS patients ($n = 15$) and controls ($n = 10$). The detection of this small molecular fragment in the urine may help nephrologists make better choices in the future treatment of FSGS patients.

15.3.5 Lupus Nephritis

LN is a common complication of systemic lupus erythematosus (SLE). In SLE, renal involvement occurs in between 15 and 75 % of patients; histological evidence of renal involvement is found in most biopsy specimens [7]. Proteomics approaches have been employed to explore noninvasive predictors of the impending relapse, relapse severity, and recovery from LN.

Zhang et al. [53] profiled the urinary proteome of LN patients in different stages of relapse using a 30-kDa cutoff filter to focus on low molecular weight proteins. Among the 27 proteins that were differentially expressed between flare intervals, 2 isoforms of hepcidin were able to predict flare onset and recovery. However, further research indicated that hepcidin was not disease specific or associated with inflammation. Somparn et al. [40] used 2-DE to profile urine samples from 5 active and 5 inactive LN patients. Two differentially excreted proteins (ZA2G and PGDS) were validated by ELISA in samples from an independent set of 78 subjects, including 30 active LN cases, 26 inactive LN cases, and 14 non-LN glomerular disease cases. ZA2G levels were elevated in the urine of patients with active LN and non-LN glomerular diseases, whereas PGDS levels were elevated only in urine from the active LN group. Urinary PGDS, not ZA2G, may thus serve as a biomarker for active LN.

In another study of the urinary proteome of children with LN [42], investigators used SELDI-TOF-MS to identify 8 peaks that differentiated patients with active nephritis from remitters and controls. These peaks had an area under the AUC of ≥0.9 for the diagnosis of active nephritis; thus, this approach appears promising for this particular group of patients.

Wu et al. [48] screened the levels of ~280 molecules in urine samples from 3 healthy individuals and 5 patients with SLE. Elevated angiostatin levels were observed and validated in an independent cohort of SLE patients ($n = 100$) by ELISA. Urine angiostatin was significantly increased in active SLE compared to inactive SLE, as was further confirmed by an ROC curve analysis with an AUC value of 0.83. However, correlation analysis of the urine angiostatin levels and renal morphological changes indicated that urine angiostatin was strongly associated with the renal pathology chronicity index but not with the activity index.

These urinary proteomics studies have revealed the potential of a urine protein panel as a noninvasive biomarker panel for distinguishing the disease activity of LN. However, the specificity and sensitivity of these markers remain inferior to that of traditional markers (such as complementary levels) and require further study, optimization, and modification.

Table 15.1 lists some urinary proteomics studies in chronic kidney diseases.

Table 15.1 Urinary proteomics studies in chronic kidney diseases

Authors	Type of disease	Participants	Method	Identified proteins
Rossing et al. [38]	DN	305 individuals	CE-MS	A model that included 65 regulated genes correctly identified diabetic nephropathy with 97 % sensitivity and specificity
Alkhalaf et al. [2]	DN	148 DM patients with albuminuria 83 DM patients without albuminruai	CE-MS	The "DN model"[a] for DN showed 93.8 % sensitivity and 91.4 % specificity, with an AUC of 0.948 (95 % CI 0.898–0.978)
Jin et al. [18]	DN	43 diabetes patients with microalbuminuria 43 diabetes patients without microalbuminuria	iTRAQ and 2DE/ Western blot/MRM	alpha-1-antitrypsin, alpha-1-acid glycoprotein 1 precursor, and prostate stem cell antigen, which had AUC values >0.8, are good biomarker candidates, and the AUC value was improved to 0.921 on combining the 3 proteins
Park et al. [27]	IgAN	13 patients with IgAN 12 healthy controls	2-GE	59 proteins were differentially expressed
Yokota et al. [52]	IgAN	17 patients with IgAN 10 healthy controls	2-D DIGE	10 proteins (albumin, transferrin, α1-antitrypsin, β-globin, α1-globin, carbonic anhydrase I, cystatin C, retinol-binding protein 4 and 1-microglobulin) were differentially expressed[b]
He et al. [15]	IgAN	56 patients with IgAN[c] 14 healthy controls	MALDI-TOF-MS	21 peaks distinguished mild and severe groups[d] 50 peaks distinguished mild and normal groups[e] 50 peaks distinguished severe and normal groups[f]

(continued)

Table 15.1 (continued)

Authors	Type of disease	Participants	Method	Identified proteins
Rocchetti et al. [36]	IgAN	18 patients with IgAN 20 healthy controls	2-D PAGE and nano-HPLC-ESI-MS/MS	Among patients with IgAN, kininogen, ITI-HC1 and transthyretin levels were different in responders and nonresponders to ACE inhibitors Low levels of urine kininogen predicted inadequate or absent clinical response to ACE inhibitors in 20 patients with biopsy-proven IgAN
Ngai et al. [26]	MN	Control rats Rats with PHN assessed at postinduction days 0,10, 20, 30, 40 and 50[g]	2D-PAGE	37 differentially expressed proteins across all time points
Piyaphanee et al. [2]	SRNS	19 SRNS 15 SSNS 10 controls	SELDI-TOF-MS	The α1-B glycoprotein was only present in 7 of 19 patients with SRNS; but absent in all SSNS and controls and associated with lower GFR.
Varghese et al. [44]	FSGS	32 patients with FSGS, LN, MN, or DN	2-DE	The urine proteins panel could distinguish different proteinuria diseases with sensitivity ranged from 75 to 86 %, and specificity ranged from 92 to 67 %
Zhang et al. [53]	LN	5 class III LN patients 11 class IV LN patients 3 class V LN patients	SELDI-TOF MS	27 protein irons showed significant differential expression between specific flare intervals of LN
Somparn et al. [40]	LN	5 active LN patients 5 inactive LN patients	2-DE	prostaglandin H 2 D-isomerase was only elevated only in the urine of the active LN group

(continued)

Table 15.1 (continued)

Authors	Type of disease	Participants	Method	Identified proteins
Suzuki et al. [42]	LN	32 pediatric LN patients 11 juvenile idiopathic arthritis patients as control	SELDI-TOF-MS	8 proteins with peaks at m/z of 2.7, 22, 23, 44, 56, 79, 100, and 133 kDa were changed in the LN patients compared with non-LN patients

[a] The DN module contains 65 genes from Rossing et al. [38] study
[b] All except 1-microglobulin were higher in patients with IgAN than controls
[c] Of whom 23 had a severe and 33 had a mild presentation
[d] For a subgroup of 10 peaks selected as biomarkers, sensitivity was 90.48 % and specificity 96.77 %
[e] For a subgroup of 10 peaks selected as biomarkers, sensitivity was 93.55 % and specificity 85.71 %
[f] For a subgroup of 20 peaks selected as biomarkers, sensitivity was 100 % and specificity 92.86 %
[g] 6 mice per group

Abbreviations *DM* diabetes mellitus; *DN* diabetic nephropathy; *iTRAQ* Isobaric tags for relative and absolute quantification; *MRM*, multiple reaction monitoring; *CE-MS* capillary electrophoresis coupled with mass spectrometry; *FSGS* focal segmental glomerulosclerosis; *MN* membranous nephropathy; *LN* lupus nephritis; *PHN* Heymann nephritis; *SRNS*, steroid-resistant nephrotic syndrome; *SSNS* steroid-sensitive nephrotic syndrome; *GFR* glomerular filtration rate; *ACE* angiotensin-converting enzyme; *DIGE* difference gel electrophoresis; *GE* gel electrophoresis; *HPLC-ESI* high performance liquid chromatography with electrospray ionization; *SELDI* surface-enhanced laser desorption/ionization; *IgAN* IgA nephropathy; *LC*, liquid chromatography; *MALDI-TOF* matrix-assisted laser desorption/ionization time-of-flight; *MS* mass spectrometry; *MS/MS* tandem mass spectrometry; and *PAGE*, polyacrylamide gel electrophoresis

15.3.6 Acute Kidney Injury

Acute kidney injury (AKI) represents a common and devastating problem in clinical medicine. The incidence of AKI varies from 5 % of hospitalized patients to 30–50 % of patients in intensive care units. Despite significant improvements in therapeutics, evidence suggests that the incidence of AKI is increasing at an alarming rate, and the associated mortality and morbidity have remained high despite improvements in clinical care [46, 49, 51]. A major reason for this high mortality and morbidity is the lack of early biomarkers for AKI, resulting in an unacceptable delay in the initiation of therapy. In addition, convenient biomarkers are urgently needed to distinguish between the various etiologies of AKI and to predict its clinical outcomes. Fortunately, the application of proteomics research to human and animal models of AKI has uncovered several novel biomarkers.

Significant efforts have been made to develop an early diagnostic biomarker for AKI in the hope that the early identification of renal injury will enable more effective therapeutic interventions. Ho et al. [16] used SELDI-TOF/MA to determine urinary proteomic profiles at different time points following coronary artery bypass grafting (CABG) operations. The active 25-amino acid form of hepcidin (hepcidin-25) was found to be dominantly elevated in postoperative non-AKI urine samples compared with AKI samples. This biomarker was further validated in an independent cohort of 338 patients [17]. The log10 hepcidin-25/Cr ratio reached a sensitivity of 68 % and a specificity of 68 %, with an AUC of 0.80 for the avoidance of AKI and a negative predictive value 0.96. Areeger et al. [3] collected urine samples from 36 patients after cardiopulmonary bypass surgery. They compared the urinary proteomes of patients with and without AKI on the first postoperative day. After the operation, inflammation-associated (zinc-α-2-glycoprotein, leucine-rich α-2-glycoprotein, mannan-binding lectin serine protease 2, basement membrane-specific heparan sulfate proteoglycan, and immunoglobulin kappa) or tubular dysfunction-associated (retinol-binding protein, adrenomedullin-binding protein, and uromodulin) proteins were found to be differentially regulated. Zinc-α-2-glycoprotein and a fragment of adrenomedullin-binding protein were decreased in patients with AKI. The decreased excretion of zinc-α-2-glycoprotein in patients with AKI was confirmed by Western blot and ELISA in an independent cohort of 22 patients with and 46 patients without AKI. Zinc-α-2-glycoprotein is thus a potentially useful predictive marker for AKI after cardiopulmonary bypass surgery.

In the last 10 years, urine neutrophil gelatinase-associated lipocalin (NGAL, also known as lcn2) has become one of the most important predictive biomarkers of AKI. NGAL is one of the earliest and most robustly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models. Indeed, the NGAL protein is easily detected in urine soon after AKI [24, 25, 45]. However, NGAL measurements may be influenced by a number of coexisting variables, such as preexisting renal disease and systemic or urinary tract infections [12]. Research to explore more accurate AKI predictive biomarkers is ongoing. Areeger et al. [4] collected urine on the first day of AKI in critically ill patients; 12 patients with an early recovery and

12 matching patients with late/non-recovery were selected, and their proteomes were analyzed by gel electrophoresis and mass spectrometry. A total of 8 prognostic candidates were identified. Subsequent ELISA quantification demonstrated that IGFBP-7 was the most potent predictor of renal recovery. IGFBP-7 and NGAL, a traditional AKI biomarker, were chosen for further analyses in an independent verification group of 28 patients with AKI and 12 control patients without AKI. The comparative analysis indicated that IGFBP-7 and NGAL were significantly upregulated in the urine of AKI patients, which in turn predicted the mortality (IGFBP-7: AUC 0.68; NGAL: AUC 0.81), recovery (IGFBP-7: AUC 0.74; NGAL: AUC 0.70), and severity (IGFBP-7: AUC 0.77; NGAL: AUC 0.69) of AKI. The levels of these proteins were also associated with AKI duration. IGFBP-7 was a more accurate predictor of renal outcome than NGAL. Thus, IGFBP-7 is a novel prognostic urinary marker that warrants further investigation.

Urinary proteomics provide a novel method for identifying early diagnostic and prognostic biomarkers of AKI. This technique can be integrated with and is complementary to traditional hypothesis-driven approaches. Moreover, this technique provides an additional armamentarium for discovery-based biomarker studies and can provide novel insights into the underlying pathophysiology of AKI, which may ultimately lead to the identification of novel therapeutic targets.

15.4 Limitations and Future Perspectives

Kidney disease has been the subject of a number of urinary proteomics studies. This research has greatly improved our understanding of the mechanisms of various kidney diseases and has provided alternative biomarkers for classification, diagnosis, and response prediction. However, several limitations have hampered the development of this approach and the translation of results to clinical applications.

First, there are challenges in the standardization of urine collection, preparation, and storage in urinary proteomics. The quality and quantity of urine proteins are affected by diet and exercise, and thus, sample collection under stable conditions is critical for the reliability and comparability of urinary proteomics results. Moreover, the storage, preparation, and analysis of urine samples may also affect the profiling. Standardization of these techniques is required to obtain more reliable proteomics data. Although an international normal urine collection protocol has been developed by the European Kidney and Urine Proteomics (EuroKUP) group and the Human Kidney and Urine Proteome Project (HKUPP) (http://www.hkupp.org), there are still no globally acceptable guidelines for urine sampling with mass proteinuria [23].

Second, compared with transcriptomic and genetic studies, urinary proteomic data sets for kidney diseases, particularly for glomerular diseases, are scarce, primarily due to the limited technology that is available for this type of study. Proteinuria is a common clinical manifestation of many kidney diseases, but severely high levels of urinary proteins complicate proteomic data collection. Thus, the technology required for pre-MS handling of samples is much more important for

proteinuria proteomics than for normal urine analysis. Unfortunately, the study of pre-MS handling for proteinuria proteomics has received much less attention than for serum proteomics. Strengthening efforts to improve pre-MS handling will benefit future biomarker discovery for kidney diseases.

Third, a lack of knowledge about the molecular mechanisms of kidney diseases poses a major challenge for detecting biomarkers through urinary proteomics. To date, most kidney diseases have been diagnosed by histological changes. Many kidney diseases, such as IgAN and FSGS, are molecularly heterogeneous diseases, which complicates the analysis of the primary data in urinary proteomics studies. Due to the biological variability and complex pathophysiology of kidney disease, urinary proteomics studies that have attempted to identify a single biomarker for kidney disease have all failed.

Furthermore, the sample size of most published studies has been small, which limits the data interpretation and predisposes the analyses to multiple testing biases. To organize a large-scale urinary proteomics study, the development of national and international consortia is required to promote strict disease classification criteria, clear criteria for the recruitment of patients into prospective cohorts, and standardized protocols for the collection of samples and detailed clinical data.

The ultimate aim of the field of urinary proteomics is to further characterize the molecular mechanisms underlying kidney diseases and to facilitate the development of improved biomarkers for the diagnosis and prediction of the therapeutic response of various kidney diseases. This is a systemic approach, and the collaborative efforts of a multidisciplinary team of physicians, molecular biologists, statisticians, and systems biologists with computer science and mathematics backgrounds are therefore needed.

There are >1,500 proteins in normal urine. Changes in these proteins reflect physiological and pathological changes in the kidney. While nephrologists have made excellent clinical diagnostic and prognostic use of albuminuria and many other urinary proteins, it is now time to delve much deeper into the urinary proteome to maximize its incredible diagnostic and prognostic potential.

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Chapter 16
Dynamic Changes of Urinary Proteins in Focal Segmental Glomerulosclerosis Model

Mindi Zhao

Abstract Compare to blood, which has mechanisms to maintain homeostasis, urine is more likely to reflect changes in the body. As urine accumulates all types of changes, identifying the precise cause of changes in the urine proteome is challenging and crucial in biomarker discovery. To reduce the confounding factors to minimal, some studies used animal model resembling human diseases. This chapter highlights the importance of animal models and introduces a strategic research which focused on adriamycin-induced nephropathy. In this study, urine samples were collected at before adriamycin administration and days 3, 7, 11, 15, and 23 after, urinary proteins were profiled by LC-MS/MS. Of 23 changed proteins with disease development, 13 proteins were identified as stable in normal human urine, meaning that changes in these proteins are more likely to reflect disease. We think this stage-dependent dynamic changes of urine proteome in animal models will help to support the role of urine as key source in biomarker discovery especially in kidney diseases and help to identify corresponding biomarkers for clinical validation.

Keywords Animal model · Confounding factors

Biomarkers represent measurable changes associated with a physiological or pathophysiological process. In contrast to the blood, which has mechanisms to maintain a homeostatic internal environment, urine is more likely to reflect changes in the body and can be collected noninvasively [1]. Due to the site of the formation and regulation of urine, the urine proteome has been widely investigated in studies of kidney, bladder, and prostate diseases [2]. As urine accumulates all types of changes, identifying the precise cause of changes in the urine proteome is challenging and crucial in biomarker discovery. The advantages of using animal models are the following: (1) reducing the effects of genetic and environmental factors on the urine proteome as much as possible; (2) knowing the exact time of disease

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occurrence, which is helpful in the identification of biomarkers for each stages including early detection; and (3) avoiding the effects of medications on the urine proteome because therapeutic measures for patients are inevitable.

Using animal model to find clues and validate them in clinical samples may be a good way to discover biomarkers in urine [1]. Some stage-specific methods have been used in some animal model studies, and most of them using two-dimensional gel electrophoresis coupled to MALDI-TOF mass spectrometry, for example, streptozotocin-induced diabetic nephropathy in rats [3] and rat models of passive Heymann nephritis [4]. As two-dimensional gel electrophoresis is time-consuming, essentially non-quantitative and limited peptides could be identified [5]. We mainly introduce an adriamycin-induced rat model study that observed dynamic changes of urinary proteins by LC-MS/MS.

Young Sprague-Dawley male rats were given a single intravenous injection of ADR (5 mg/kg). Urine samples were collected at days 0 (before injection), 3, 7, 11, 15 and 23 after the rats were placed in metabolic cages individually. The urinary protein to creatinine ratios were measured to determine the proteinuria levels. As shown in Fig. 16.1, the ratios increased. To confirm the successful induction of FSGS, the histological characteristics of the rat kidneys on days 23 and 50 were examined.

By label-free quantitative and statistical analyses, 23 proteins met the following conditions: (1) compared with day 0, max fold change >2 in each rat and (2) p value < 0.05. Among the 23 changed proteins, 20 proteins were annotated as glycoproteins in the UniProt database, 12 proteins shared an overall increasing trend in relative abundance, and 9 proteins shared an overall decreasing trend. Three trends were observed in these candidate biomarkers during ADR-induced nephropathy progression. The first was a gradual increase, with examples including afamin and ceruloplasmin. The second was a gradual decrease, with examples including cadherin-2 and aggrecan core protein. The third, which includes fetuin-B and beta-2-

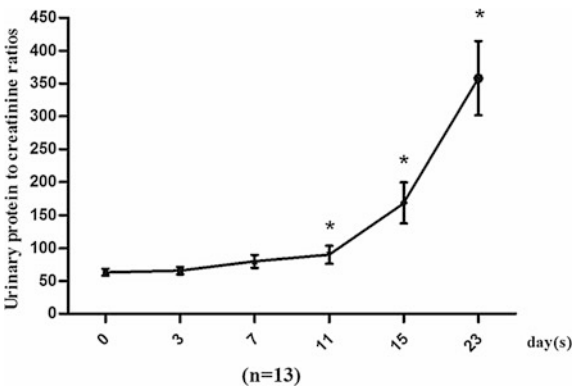

Fig. 16.1 Dynamic changes in ADR-treated rats. Urinary protein to creatinine ratios in six phases of ADR-treated rats. The data are expressed as the mean ± standard deviation ($n = 13$, * $p < 0.05$ for Experiment vs. Control). The paired t test was used to assess the significance of the differences between groups [10]

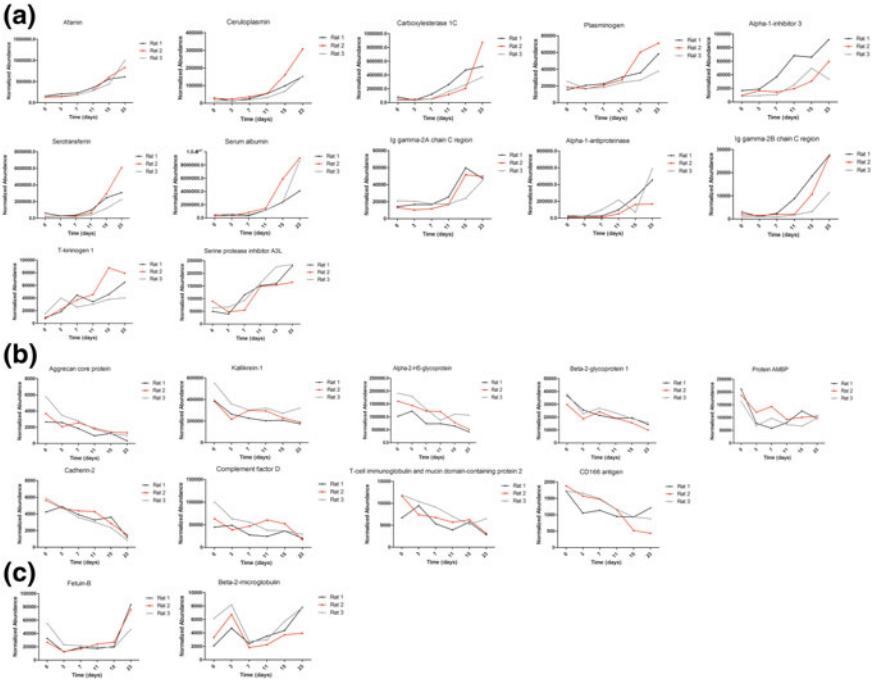

Fig. 16.2 Expression of candidate urine biomarkers of ADR-induced nephropathy during six stages. The *x* axis represents different stages; the *y* axis represents the normalized abundance identified by the LC-MS Progenesis software [10]

microglobulin, was early changes with distinct patterns. The time-dependent changes of 23 representative proteins from three rats are shown in Fig. 16.2.

At the early stage, such as days 3 and 7 after the ADR injection, proteinuria was not obvious. However, several proteins, such as fetuin-B, AMBP, and kallikrein-1, were decreased during this phase. These proteins may be good candidates for the early detection.

Candidate biomarkers demonstrated in animal models are more valuable if they can be confirmed in humans. The changed proteins were converted to their corresponding human orthologs using Ensembl Gene ID(s) by Ensembl BioMart (http://asia.ensembl.org/biomart/martview) as described [6, 7]. Of 23 identified changed proteins, 20 have human orthologs, including the seven validated by Western blot analysis. A previous study compared the kidney input (plasma) and output (urine) proteomes and divided urinary proteins into 3 categories, the plasma-only subproteome, the plasma-and-urine subproteome, and the urine-only subproteome [8]. To further analyze the functions of these candidate biomarkers, these changed proteins were compared with the human plasma proteome, human urine proteome, and kidney origin proteome. The human proteome data were downloaded from the Healthy Human Individual's Integrated Plasma Proteome (HIP^2) [7, 9], the human

Table 16.1 Comparison of human orthologs of candidate biomarkers with the normal human urine proteome, plasma proteome, and kidney origin proteome [10]

Corresponding human protein ID	Protein name	Plasma proteome	Urine proteome	Kidney origin proteome	Stable protein	Candidate biomarkers
P01009	Alpha-1-antiproteinase	Yes	Yes	Yes	Yes	FSGS [14]
P43652	Afamin	Yes	Yes	Yes	Yes	Diabetic nephropathy [15]
P02768	Serum albumin	Yes	Yes	Yes	Yes	Adriamycin nephropathy mice model [16]
P00450	Ceruloplasmin	Yes	Yes	Yes	Yes	Diabetic nephropathy [17]
P01042	Kininogen-1	Yes	Yes	Yes	Yes	Adriamycin nephropathy rat model [18]
P02787	Serotransferrin	Yes	Yes	Yes	Yes	Adriamycin nephropathy mice model [16]
P23141	Carboxylesterase 1	Yes	Yes	No	No	None
P01861	Ig gamma-4 chain C region	Yes	Yes	No	No	Kidney calculi [19]
P00747	Plasminogen	Yes	Yes	No	Yes	Acute appendicitis [20]
P02765	Alpha-2-HS-glycoprotein	Yes	Yes	Yes	Yes	Diabetic nephropathy [15]
P02760	Protein AMBP	Yes	Yes	Yes	Yes	FSGS [14]
Q96D42	T-cell immunoglobulin and mucin domain-containing protein 1	No	No	No	No	Renal cell carcinoma [21], acute renal failure [22]
P19022	Cadherin-2	Yes	Yes	No	Yes	Ureteropelvic junction obstruction [23]
Q6PID9	Aggrecan core protein	No	Yes	No	No	None
P00746	Complement factor D	Yes	Yes	No	No	Dents disease [4, 24]
P02749	Beta-2-glycoprotein 1	Yes	Yes	No	Yes	Dents disease [24]
Q13740	CD166 antigen	Yes	No	No	No	None
P06870	Kallikrein-1	No	Yes	No	Yes	None
P61769	Beta-2-microglobulin	Yes	Yes	Yes	Yes	Renal interstitial inflammation [25]
Q9UGM5	Fetuin-B	Yes	Yes	Yes	No	None

urine proteome data were acquired from previous studies [11–13], and the kidney origin proteome data were acquired from a kidney perfusion study [7]. The human orthologs of the changed proteins and their relationships with the human plasma, urine, and kidney origin proteomes are shown in Table 16.1. Most changed proteins exist in the normal human plasma proteome (17/20) and urine proteome (18/20); however, the CD166 antigen was detected only in the plasma proteome, kallikrein-1 was detected only in the normal urine proteome, and 10 proteins were detected in the kidney origin proteome. In addition, the urinary proteome is largely affected by individual factors, but biomarkers should be applicable to most people, in other words, changes in the stable contents of the healthy human urinary proteome are more likely to become biomarkers [26]. A total of 560 proteins were considered stable in healthy human urine [27]. Thirteen out of twenty candidate biomarkers in this study could be found among the stable proteins, indicating that these candidate biomarkers were more useful for clinical diagnoses as they are not affected by individual differences and are not time dependent.

Candidate biomarkers identified by progressive ADR-induced nephropathy were compared with a manually curated human and animal urinary protein biomarker database [28]. Five high-abundance urinary proteins, including albumin, serotransferrin, and kininogen-1, had been present in higher levels than in the normal condition in previous FSGS studies [14, 16, 18], which is consistent with the results of this study. The other 15 proteins were not found to be related to FSGS. Some proteins were found to be candidate biomarkers for other glomerular diseases; for example, elevated afamin expression has been reported in some diabetic nephropathies. The similarities may due to a common pathway of different glomerular diseases at later stages [29]. Thus, these diseases can be differentiated using changed proteins, especially those significantly decreased proteins in the early stage, such as fetuin-B and AMBP. Some candidates also appeared in other renal diseases, whereas the opposite trends were observed in different diseases. For example, complement factor D and beta-2-glycoprotein 1 were higher in Fanconi syndrome [30] but lower in ADR-induced nephropathy.

As the same urinary proteins appeared to be modulated in several renal diseases, for example, albumin was increased in both diabetic nephropathy and IgA nephropathy. Thus, it may be difficult to provide an accurate diagnosis using a single biomarker; a panel of urinary proteins may be more specific and more sensitive. We hope this strategy will help to support the role of urine as key source in biomarker discovery especially in kidney diseases and help to identify biomarkers for clinical validation.

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Chapter 17
Using Isolated Rat Kidney to Discover Kidney Origin Biomarkers in Urine

Lulu Jia

Abstract The use of targeted proteomics to identify urinary biomarkers of kidney disease in urine can avoid the interference of serum proteins. It may provide better sample throughput, higher sensitivity, and specificity. Knowing which urinary proteins to target is essential for targeted proteomics. In perfusates, there were proteins not found in normal human urine which may become biomarkers with zero background. There were proteins not found in normal human plasma which will not be influenced by other normal organs and will be kidney specific. When compared with existing candidate biomarkers, over 90 % of the kidney origin proteins in urine identified in this study have not been examined as candidate biomarkers of kidney diseases.

Keywords Kidney origin proteins · Biomarker

17.1 Introduction

The identification of urinary biomarkers for kidney diseases may be easier to accomplish than the identification of biomarkers for other diseases such as cancer. The current pipeline of biomarker identification comprises two separate stages: discovery and validation [1]. A comprehensive, profiling-based differential proteomics methods, which have limited sample throughput because of their prolonged sample analysis, are generally used in the discovery phase [2]. However, kidney diseases usually produce proteinuria, which is manifested by the appearance of high abundant blood proteins in the urine, due to the breakdown of filtration and reabsorption functions of the kidney. This tends to obscure the appearance of proteins

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derived from kidney tissue, which would provide the most sensitive and specific markers for tissue injury and mechanism. Profiling is easily influenced by the preferential detection of highly abundant plasma proteins in the urine, which exhibit similar changes under many different renal conditions and lose specificity [3]. Therefore, the identification efficiency of urinary biomarkers for kidney disease has been severely restricted.

Advances in targeted proteomic technologies simultaneously allow the quantification of hundreds of proteins with better sample throughput, high sensitivity, and high specificity [4–6]. The disadvantages of profiling methods can be avoided by using targeted proteomic technologies in the discovery phase. Targeting the kidney origin proteins in urine using target proteomics directly in the discovery phase will facilitate the kidney disease biomarker discovery. This needs us to identify kidney origin proteins in urine first.

Analysis of kidney origin proteins in urine should exclude proteins present in urine as the result of the ultrafiltration of plasma. Isolated kidney perfusion is a classic technique that has been widely used for the study of renal physiology, pharmacology, and pharmacokinetics [7]. A perfused isolated kidney can maintain approximately normal physiological functions for more than two hours when it is circularly perfused with artificial blood-free perfusion fluids [7]. Here, we used a modified isolated rat kidney perfusion model to analyze the kidney origin proteins present in the urinary tract [8].

17.2 Isolated Rat Kidney Perfusion Model

17.2.1 Perfusate Preparation

Modified Krebs–Henseleit buffer (4.7 mM KCl, 1.2 mM KH_2PO_4, 2.1 mM $MgSO_4$, 117 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11 g/L glucose) was used as a perfusate with 60 g/L clinical grade dextran added as an oncotic agent [9]. Twenty amino acids were combined in solution to achieve the following concentrations: 2 mM alanine, 0.5 mM arginine, 0.2 mM asparagine, 0.2 mM aspartate, 0.5 mM cysteine, 0.5 mM glutamate, 2 mM glutamine, 2.3 mM glycine, 0.24 mM histidine, 0.3 mM isoleucine, 0.4 mM leucine, 1 mM lysine, 0.33 mM methionine, 0.32 mM phenylalanine, 0.31 mM proline, 1 mM serine, 0.24 mM threonine, 0.07 mM tryptophan, 0.2 mM tyrosine, and 0.33 mM valine [10]. The perfusate was filtered through a 0.45-μm filter and equilibrated with a mixture of oxygen and carbon dioxide (95 % O_2/5 % CO_2) for at least 2 h prior to use. The perfusate was used within 6 h of preparation, and the pH of the solution was adjusted to 7.4 with hydrochloric acid prior to use.

17.2.2 Surgical Procedures

The surgical technique was based on methods described previously [7]. Briefly, rats were anesthetized via an intraperitoneal injection of sodium pentobarbitone (40 mg/kg). A midline laparotomy incision was made from the pelvis to the sternum on the animal. The right ureter was ligated immediately proximal to the bladder. A solution of mannitol (150 mg) and heparin (100 U) in 1 ml normal saline was injected into the venae saphena magna. The right ureter was cannulated with a 24G vein detained needle to collect the urine. The aorta was clamped distal to the right renal artery and cannulated with an 18G vein detained needle a few millimeters distal to the renal artery in the retrograde direction. The superior mesenteric artery and the aorta proximal to the right renal artery were ligated so that the artificial perfusates could flow into the right kidney. The infusion tube was inserted into the inferior vena cava from the near heart end to arrive at the right renal vein to drain the perfusates. After the completion of the surgery, the rat was transferred into a small incubator to maintain a temperature of 37 °C.

17.2.3 Perfusion Design

The isolated kidney was perfused in situ. Perfusates, aerated with a mixture of 95 % O_2/5 % CO_2, were sequentially pumped through the in-line filter, the bubble trap, and a 37 °C warming system to enter the isolated kidney. The intrarenal perfusion pressure was maintained at 110 ± 5 mm Hg by adjusting the flow rate of the rotary pump, which was continuously monitored using a manometer and was corrected for the intrinsic pressure of the apparatus.

The isolated kidney was first perfused in single-pass perfusion mode for 10–15 min, allowing the kidney to pre-equilibrate and flushing out the residual blood in the kidney. The mode was then changed to recirculating mode with a recirculating perfusion medium volume of 300 ml and duration of 40 min. The kidney was perfused with oxygen-supplemented perfusion medium during this period. The urine was collected at 10-minute intervals during this stage. When the first stage was complete, 2 ml of the perfusates and perfusion-driven urine was centrifuged at 12,000×*g* for 10 min at 4 °C to determine whether there were red blood cells in the perfusates, and the cell debris in the perfusion-driven urine was evaluated by macroscopic observation. Only isolated perfused kidneys that were negative for both of the above inspections were considered to be successful preparations and proceeded to the next stage. In the next stage, another 300 ml of fresh perfusion medium was used to perfuse the kidney for another 40 min under the same conditions, except that the perfusion medium was not supplemented with oxygen. The perfusion pressure and flow rate were recorded at 10-min intervals over both stages of the experiments. When the experiment was complete, the perfusion-driven urine with and without oxygen supplementation was collected and prepared for analysis.

17.2.4 Preparation of Proteins in Perfusion-Driven Urine

For the comprehensive analysis of the perfusion-driven urine proteome with oxygen supplementation, the collected urine was loaded onto 20 × 12 cm, 12 % polyacrylamide gels for separation. After the gels were stained with colloidal Coomassie blue, the lanes were excised into 26 1–2-mm-wide slices and subjected to in-gel digestion.

For the comparison of perfusion-driven urine proteomes with and without oxygen supplementation, the perfusion-driven urines were firstly transferred into a 3-kDa cutoff centrifugal column to reduce the volume to 250 μL, and then were loaded onto a custom 12 % acrylamide gel with large wells for SDS-PAGE analysis, respectively. Electrophoresis was stopped when the proteins were concentrated in bands between the stacking and resolving gels according to the prestained protein marker. The protein bands were cut into small pieces and subjected to in-gel digestion. The peptides that were extracted from these gel pieces were prepared for LC-MS/MS analysis.

17.3 Comprehensive Profiling of the Perfusion-Driven Urine Proteome Using SDS-PAGE-LC-MS/MS

The proteins present in perfusion-driven urine were separated using SDS-PAGE. Lanes were cut into 26 slices. After digestion of the proteins with trypsin, each slice was analyzed using LC-MS/MS. MS/MS files acquired from each fraction were merged, and the proteins were identified by performing a database search using MASCOT. Two perfusion-driven urine samples acquired from two independent isolated rat kidneys were analyzed using different mass spectrometry platforms, an LTQ Orbitrap Velos platform and a high-speed TripleTOF 5600 system. A total of 1,782 and 3,025 proteins, respectively, were identified with more than two distinct peptides. There are 1,402 proteins common to both samples. The proteins common to both methods were subjected to subsequent analysis.

17.4 Identification of Human Orthologs for the Proteins in Isolated Rat Kidney Perfusion-Driven Urine

It is typically assumed that orthologs (co-orthologs) retain similar functions between species [11, 12]. Human orthologs for proteins in the isolated rat kidney perfusion-driven urine were identified. There is currently no “gold standard” for identifying a complete set of orthologs between two species [13]. Different orthologous protein databases use the different orthology prediction methods and thus yielded different and overlapping results. In Paranoid [14], OrthoMCL-DB [15], Homogene [16], Ensembl Compare [17], and 122.R_norvegicus.orthologues

(http://www.ebi.ac.uk/) are used to search for human orthologs of identified proteins. Human orthologs that were identified from the same rat proteins by at least two databases were compiled, resulting in the pairing of 1,234 of the 1,402 rat proteins to 1,233 human orthologs, which account for 1,278 human orthologous genes.

17.5 Comparison of Human Orthologs for Proteins in the Perfusion-Driven Urine with Kidney Protein Expression Data, the Normal Human Urine Proteome, and the Plasma Proteome

While the human orthologs expressed in the kidney are likely to be kidney origin proteins found in the normal human urine, the human orthologs not expressed in the kidney may be residual interstitial fluid proteins and/or may be plasma proteins that were absorbed by the kidney. To identify kidney origin proteins in urine, the human orthologs were compared with human kidney expression data. Data detailing the expression of human kidney proteins were acquired from the Human Protein Atlas Database [18]. Expression data from 12,260 human kidney genes were acquired.

The human orthologs of rat perfusion-driven urine proteins were also compared with normal human urine proteome (including the urinary exosome proteome) and human plasma proteome to determine which human orthologs have been identified in normal human urine and plasma, respectively. For the normal human urine proteome, three large-scale datasets were collected from previous studies [19–21] and one large-scale dataset from one team in our institution. For the normal human urinary exosome proteome, three large-scale human urinary exosome datasets were collected from previous studies [22–24]. For the normal human plasma proteome, the largest human plasma proteome dataset was acquired from an online database, Healthy Human Individual's Integrated Plasma Proteome (HIP2) [25].

For easier comparison, the protein identifiers in different datasets and the kidney expression genes were standardized. Ensembl BioMart (http://asia.ensembl.org/biomart/martview) was used to transform all of the different protein identifiers to Ensembl Gene ID(s). The different proteome datasets were compared at the gene level. All of the human urine proteins, urinary exosome proteins, and plasma proteins from different datasets were pooled together. This process resulted in 5,225 non-redundant genes in human urine, 3,416 non-redundant genes in the human urine exosome, and 9,706 non-redundant genes in human plasma. The genes in human urine and the urine exosome were pooled, which resulted in 6,084 non-redundant genes in normal human urine and the urinary exosome.

The 1,233 human orthologs, which account for 1,278 human orthologous genes, were compared at the gene level with human kidney gene expression, the pooled human urine and urinary exosome proteome, and the human plasma proteome (Fig. 17.1).

Of the 1,278 genes, 982 were expressed in the kidney. These genes corresponded to 981 human orthologs. The 981 human orthologs with gene expression in the kidney were considered to be potential human kidney proteins in urine. Of the 981 human orthologs, 613 had been identified both in the urine (including urinary exosome) proteome and the plasma proteome; 240 had only been identified in the urine (including urinary exosome) proteome but not in the plasma proteome; 71 had only been identified in the plasma proteome but not in the urine (including urinary exosome) proteome; and 57 had not been identified in either the urine (including urinary exosome) proteome or the plasma proteome (Fig. 17.1).

There are a total of 128 human orthologs (57 plus 71) that were expressed in the kidney but were not present in normal urine (including the urinary exosome). They are potential biomarkers with zero background in pathological conditions. There are a total of 297 human orthologs (57 plus 240) that were expressed in the kidney but were not present in the plasma. They are likely not influenced by other normal organs, including the plasma and therefore have the potential to specifically reflect functional changes in the kidney. The 57 human orthologs could be sensitive markers because they were not present in normal urine or the urinary exosome and were not influenced by other normal organs, including plasma.

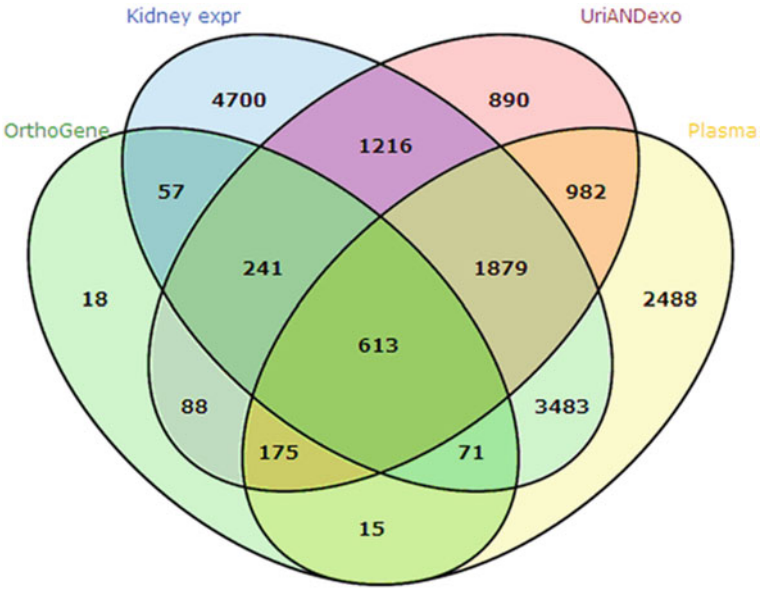

Fig. 17.1 The human orthologs identified from the rat proteins in perfusion-driven urine were compared with human kidney expression data (*Kidney expr*), the pooled human urine and urinary exosome proteome (*UriANDexo*), and the human plasma proteome (*Plasma*). The protein identifiers were standardized using the Ensembl Gene ID(s). The comparison was performed at the gene level [28]

17.6 Comparing the Ranking of Human Kidney Origin Proteins in the Normal and Perfusion-Driven Urine

One large-scale dataset of the human normal urine proteome was collected from one team in our institution. They used the same MASCOT search engine as in this study. The Exponentially Modified Protein Abundance Index (emPAI), which offers approximate, label-free, relative quantitation of the proteins in a mixture based on protein coverage by peptide matches, has been incorporated into the MASCOT search engine [26]. Therefore, each identified urine protein had an emPAI value, which can be used to approximately estimate the absolute protein contents in urine.

Of the 981 human orthologs that were considered to be potential human kidney origin proteins in urine, 775 were identified in this normal human urine dataset. The emPAI values of these human orthologs were extracted from the normal human urine proteome, and these proteins were sorted from most to least abundant in the normal human urine. Proteins not identified in the human urine were at the end. The order of these human orthologs approximately represents their abundance in human urine under physiological conditions.

The 981 human orthologs were paired to rat proteins that were identified in both isolated rat kidney perfusion-driven urine samples. The rat proteins corresponding to human orthologs had an emPAI value when they were identified in the perfusion-driven urine, which can be used to approximately estimate the absolute protein content in the perfusion-driven urine. The rat proteins corresponding to the human orthologs were sorted according to their emPAI values in the two perfusion-driven samples from most to least abundant in the perfusion-driven urine. Due to the correspondence between rat proteins and their human orthologs, this resulted in the reordering of the 981 human orthologs. We assume that the abundances of orthologous proteins in the human and rat samples have a certain correlation. The new order of the 981 human orthologs sorted by the abundance of their paired rat proteins in the perfusion-driven urine might approximately represent the abundance order in the pathological condition.

For a given protein, if the abundance ranking increased significantly from the normal urine to the perfusion-driven urine, expression of that protein might increase under pathological conditions compared to other proteins. The ranks of the corresponding rat proteins in the two perfusion-driven urine samples were compared first. The vast majority, 922 proteins (94 %), had ranking changes of less than 300. Therefore, a ranking change of 300 was considered to be significant. In total, 75 of the 981 human orthologs increased in rank by 300 from the normal human urine to the two perfusion-driven urine samples.

The emPAI value is only an approximate estimation of the absolute protein content in a protein mixture [26]. The degree of correlation between orthologous protein abundance was not investigated. Here, we only observed that, for the 75 human orthologs, the rank of their corresponding rat proteins increased significantly

in the perfusion-driven urine compared to their rank in the normal urine. We expect the large difference in the abundance ranking of these proteins will indicate their potential to be kidney disease biomarkers.

17.7 Comparison of the Perfusion-Driven Urine Proteomes During Perfusion with and Without Oxygen Supplementation Using LC-MS/MS

The urine proteomes from four independent isolated perfused rat kidneys during perfusion with and without oxygen supplementation were profiled using LC-MS/MS. The samples from two of the rats were profiled with the LTQ Orbitrap Velos platform, which identified 236 and 280 proteins during perfusion with oxygen supplementation and 275 and 281 proteins during perfusion without oxygen supplementation. The samples from the other two rats were profiled with the Triple-TOF 5600 platform, which identified 474 and 466 proteins during perfusion with oxygen supplementation and 511 and 527 proteins during perfusion without oxygen supplementation.

The expression of the proteins present during perfusion with oxygen-supplemented medium was compared with expression during perfusion without oxygen supplementation using the label-free quantitative method provided by the SCAFFOLD program. The expression of 39 proteins was significantly increased in all four perfusion-driven urine samples when the kidneys were perfused without oxygen supplementation ($p < 0.05$, T-test).

These 39 proteins were matched to human orthologs using the same method described above. In total, 33 human orthologs were identified. Because their corresponding rat proteins were increased in the perfusion-driven urine when the kidneys were perfused without oxygen supplementation, these 33 human orthologs were also considered to be the potential human kidney origin proteins in urine.

17.8 Comparison of the Human Kidney Origin Proteins in Urine with Previous Biomarker Studies

A total of 990 non-redundant human orthologs were generated by pooling the perfusion-driven urine proteins that are expressed in the kidney and increased in perfusion-driven urine from oxygen-deficient kidneys. These proteins are potential human kidney origin proteins in urine. Of the 990 kidney origin proteins, there are a total of 428 proteins that may be high-quality potential candidate biomarkers, including kidney origin proteins present in the perfusion-driven urine but not in normal urine, kidney origin proteins present in the perfusion-driven urine but not in

the large-scale plasma database, kidney origin proteins that are increased in perfusion-driven urine from oxygen-deficient kidneys, and kidney origin proteins that have a large increase in rank in the perfusion-driven urine compared to normal human urine.

The urinary Protein Biomarker Database was established by comprehensively compiling and manually curating the published literature [27]. A total of 343 candidate biomarkers for human kidney diseases have been collected from the Urinary Biomarker Database [27]. Compared with this database, 67 of the 990 kidney origin proteins have been studied as candidate biomarkers of kidney diseases (Fig. 17.2). Of the 428 high-quality kidney origin proteins, seven proteins have been studied as the candidate biomarkers of kidney diseases.

However, 923 (93 %) kidney origin proteins have not been studied as candidate biomarkers. Furthermore, few of the 67 kidney origin proteins that were identified as candidates in large-scale differential proteomics experiments have been examined in more detail according to the urinary biomarkers database. One reason why studies examining urinary biomarkers in kidney disease have not been conclusive might be because kidney origin proteins were not examined in detail. These 428 high-quality kidney origin proteins are potential urinary kidney disease markers that should be examined in detail. Because there are hundreds of potentially useful urinary kidney disease markers, combinations of these proteins are likely to be able to differentiate many different kidney conditions.

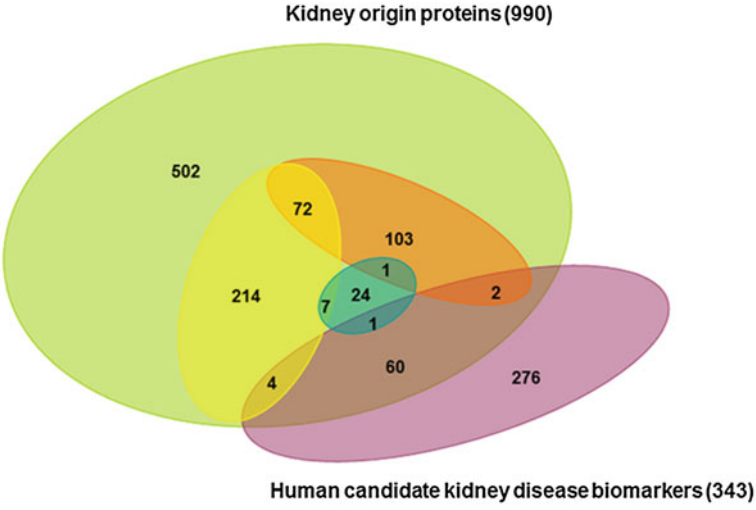

Fig. 17.2 A comparison of the identified kidney origin proteins with previously identified human candidate biomarkers of kidney disease. The *yellow oval* represents proteins present in perfusion-driven urine but not in normal human plasma. The *orange oval* represents proteins detected in perfusion-driven urine but not in normal human urine (including human urinary exosomes) or present in human urine but significantly increased in the perfusion-driven urine. The *blue oval* represents proteins with an increased level in perfusion-driven urine without oxygen supplementation compared to perfusion with oxygen-supplemented medium [28]

17.9 Conclusions and Discussion

We presented an approach to profile the isolated rat kidney perfusion-driven urine proteome, to match the identified rat proteins to human orthologs, and then the human orthologs were compared with human kidney expression data, the human urine proteome (urinary exosome proteome), and the plasma proteome. The perfusion-driven urine proteomes during perfusion with and without oxygen supplementation were also compared. Finally, we identified 990 human orthologs that were potential human kidney origin proteins in urine. We identified 428 high-quality kidney origin proteins that may become kidney disease biomarkers. The high-quality kidney origin proteins are either not present in plasma or normal urine or increased during perfusion. The kidney origin proteins identified in this study can be used to direct targeted proteomics studies in the discovery phase for kidney disease biomarkers. We recommend that the high-quality kidney origin proteins be screened first using targeted proteomics.

Isolated organ perfusates have advantages in the search for potential biomarkers, including accessibility, sensitivity, and specificity. Many proteins that are differentially expressed in tissue are not detectable in bodily fluids. Perfusates are a reflection of the proteins that are accessible in bodily fluids. The concentration of the potential biomarkers is higher in perfusates than in bodily fluids. When compared with plasma or urine, perfusates reduce the proteome complexity to facilitate protein identification. Furthermore, perfusates exclude the influence of other organs. It should be noted that a biomarker will only be fully relevant once it can be validated in the whole organism. The candidate biomarkers identified in organ perfusates should be validated in bodily fluids relevant to the disease condition of the specific organ.

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Chapter 18
Comparing Plasma and Urinary Proteomes to Understand Kidney Function

Lulu Jia

Abstract Kidney function is rarely studied in the context of blood kidney and urine as a system. Kidney can be considered as a black box, while plasma and urine proteomes closely represent the protein compositions of the input and output of the kidney. This idea provides a new approach for studying organ functions with a proteomic methodology. Because of its distinctive input (plasma) and output (urine), it is reasonable to predict that the kidney will be the first organ whose functions are further elucidated by proteomic methods in the near future.

Keywords Kidney function · Black box

18.1 Introduction

A large volume of plasma (350–400 mL/100 g of tissue per min) is filtered by the kidney to generate about 150–180 L/per day ultrafiltrate, and then, most components in the ultrafiltrate are selectively reabsorbed until less than 1 % of the ultrafiltrating volume is excreted as urine [13]. In this physiological process, the plasma proteins are filtered by the kidney. After handling by the kidney including reabsorption and secretion, the urine proteins were produced and excreted. In short, the plasma proteins enter the kidney, then the urine proteins out. The kidney can be regarded as a black box with distinct input and output proteomes. Therefore, the kidney's protein handling function can be studied by comparing plasma and urine proteomes.

The plasma proteome could be regarded as the input proteome. However, the urine proteome could not be simply regarded as the kidney output proteome due to its complicated protein sources. In addition to the kidney, urine proteins may also

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Fig. 18.1 Kidney function analysis by black box method

be derived from the urine tract and glands downstream of the kidney [9, 11]. Proteins from latter sources might complicate the kidney output. For black box analysis, all input should go into the black box, and all output should come directly out of the black box. For analysis of the kidney's protein handling function, available proteins secreted into the urine downstream of kidney were subtracted from the plasma and urine proteomes to form the effective input and output proteomes, respectively (Fig. 18.1) [3].

By comparing these modified kidney input and output proteomes, this work first aims to find which proteins are blocked or permitted to pass through and which proteins are secreted or shed from the kidney. Because these different protein handling pathways in the kidney are closely related to the form and size of individual proteins, the experimental molecular weights (MWs) of proteins are therefore important for understanding the mechanisms of protein handling by the kidney. Then, the experimental MWs of the proteins in plasma and urine are compared. For different proteins, the associations between the quantitative changes from plasma proteome to urine proteome and their MWs were also investigated.

18.2 Kidney Input Proteome and Output Proteome

The human plasma proteins were collected from the plasma proteome project (PPP) initiated by HUPO, who published a high confidence (FDR 1 %) list of 1,929 identified proteins (http://www.mcponline.org/content/suppl/2011/06/01/mcp.M110.006353.DC1/mcp.M110.006353-4.xls) including Phase I and II data, in June 2011 [Farrah et al. (http://www.mcponline.org/content/early/2011/06/01/mcp.M110.006353.long)]. The human urine proteins were collected from three large-scale datasets of the previous studies [1, 6, 7] and one large-scale dataset identified in our institution (data not published). There are only a limited number of proteins with experimental MWs information published so far in plasma and urine. In this study, the experimental MWs of the proteins were collected from the previous study [4, 8, 10].

The prostate is a secretory gland downstream of the kidney that might secrete prostatic fluid into urine, which would complicate the kidney output proteome. Human prostatic secretion proteins identified by proteomic methods were hence removed from the urine proteome and plasma proteome. A total of 114 human prostatic secretion proteins were acquired from Lin et al. [5]. Other proteins that are possibly incorporated in urine downstream of kidney were temporarily ignored due to limited knowledge at this time.

For easier comparison, the protein identifiers in different datasets were standardized. The UniProt Reference Clusters (UniRef) provide clustered sets of sequences from the UniProt Knowledgebase (http://www.uniprot.org/help/uniprotkb) [including isoforms (http://www.uniprot.org/help/canonical_and_isoforms)] while hiding redundant sequences. UniRef90 was selected as the reference database. All the protein identifiers of different datasets were mapped to UniRef90 cluster using ID mapping tool from UniProt Web site. After protein ID standardization and removal of prostatic secretion proteins, kidney input and output proteome were generated. This resulted in 1,778 non-redundant UniRef90 sequences in the plasma as the kidney input proteome and 6,093 non-redundant UniRef90 sequences in the urine as the kidney output proteome.

18.3 Kidney Function Described in Proteomic Language

The plasma-only subproteome, the plasma-and-urine subproteome, and the urine-only subproteome were generated after comparing the kidney input and output proteomes with the kidney considered as a black box. The function of the kidney can be described in itemized proteomic language as whether a particular protein is blocked, permitted to pass, or secreted/shed from the kidney. These three groups of proteins correspond to the three subproteomes.

18.3.1 Plasma-Only Subproteome

There were 351 non-redundant protein sequences in the plasma-only subproteome. Currently, there was no evidence that they were present in urine based on available proteomic data. Due to the profiling depth of the urine proteome is significantly exceeded that of the plasma proteome, this group of proteins is more reliable. These proteins are supposed to be difficult to pass through the kidney black box. There were only 4 proteins with experimental MWs, which were relatively small proteins. They were P11226 (26.3 kDa), P02745 (26.3 kDa), P20742 (51.6 kDa), and Q14289 (65.1 kDa). The theoretical MWs were in a range from 3 kDa to more than 400 kDa, while the theoretical PIs were in a range from 4 to 11.6. According to estimated concentration of the proteins in plasma provided by plasma proteome dataset, there were many moderate- or even high-abundant proteins that were present only in plasma but not in urine. Particularly, there were some high-abundant Ig proteins present only in plasma. This group included proteins that could not be filtered at the glomerular capillaries or filtered but reabsorbed completely back into the blood from the tubules.

18.3.2 Plasma-and-Urine Subproteome

There were 1,424 non-redundant protein sequences that existed in both plasma and urine. They may pass the kidney black box in various forms. There were only 15 proteins with experimental MW information available from both plasma and urine, which range from 11 to 133 kDa in plasma and 11 to 77 kDa in urine. Comparing their MWs in plasma and in urine, six proteins were within 20 % variation, suggesting that they may pass through the kidney in an intact form; four had MWs 20 % higher in plasma than in urine, and five had MWs 20 % lower in plasma than in urine. These differences reflect functions of the kidney. In addition to one protein whose theoretical MW was 3,816 kDa, the theoretical MWs of the proteins were in a range from 3 kDa to more than 600 kDa, while the theoretical PIs were in a range from 3.6 to 12.

For the kidney considered as a black box, the quantitative changes of different proteins from plasma proteome to urine proteome reflect the kidney protein handling function. Therefore, the ranking order of the plasma and urine proteins sorted by their concentrations in plasma was compared with that in urine. The estimated concentrations of the proteins in plasma were provided by plasma proteome dataset, which were used to generate their ranking order in plasma. Many urine proteins were collected from a large-scale dataset identified in our institution using MASCOT search engine (data not published). The MASCOT search engine has been incorporated the exponentially modified protein abundance index (emPAI), which offers approximate, label-free, relative quantitation of the proteins in a mixture based on protein coverage by peptide matches [2]. Each identified urine protein had an emPAI value, which can be used to approximately estimate the absolute protein contents in urine. The emPAI values of these plasma and urine proteins were extracted from the large-scale dataset. These proteins were sorted from most to least abundant. Proteins not identified in the large-scale dataset were at the end. The ranking order of these proteins approximately represents their abundance in human urine. After comparing their ranking order in plasma with that in urine, we found that the two ranking orders did not correspond well with each other. Many proteins have a significantly changed ranking order when they passed through the kidney. This suggested that the kidney performed different handling functions for the different proteins.

It is believed that proteins with a MW of <15 kDa are freely filtered in the glomeruli; proteins up to 45 kDa are quite rapidly filtered and proteins between 45 and 60 kDa only restrictedly. Plasma proteins larger than 60 kDa are not filtered through the kidney [12]. We found that some proteins with experimental MW <45 kDa exist in the plasma but have not been identified in the urine proteomic data until now. Particularly, some moderate-abundant and high-abundant proteins even they are low molecular weight were present only in plasma but not in urine. There were some possible mechanisms. For example, they might bind to larger carrier proteins or there might be some unknown mechanisms for them to be retained for an extended period in the plasma. We have also found that some

proteins with experimental MW >60 kDa had been identified both in plasma and in urine such as P35858 (experimental molecular weight 80 kDa in plasma and 77 kDa in urine) and P05155 (experimental molecular weight 91 kDa in plasma and 75 kDa in urine). These proteins might be secreted, but the passing through the glomeruli could not be ruled out. This is worth of further study. When the proteins passed through the kidney, the different quantitative changes of the proteins from plasma proteome to urine proteome reflect the different handling function of that particular protein by kidney.

18.3.3 Urine-Only Subproteome

Four thousand one hundred and eighty-one proteins were identified only in urine, but not in plasma, by proteomic methods. Proteins secreted or shed from the kidney are thought to be included in this group. Since the profiling depth of the urine proteome is higher than that of the plasma proteome, there were potentially a lot of false-positive proteins. In other words, some of the 4,181 proteins might exist in plasma but were missed, so they may have been determined to belong to plasma-and-urine subproteome instead of urine-only one.

18.4 Discussion and Perspective

Since mass spectrometry-based proteomics was founded, body fluid proteomes, such as plasma, urine, tear, and cerebrospinal fluid, have been profiled by many groups. All of the body fluids interact with each other and the organs and finally collectively contribute to form a dynamic system in the body. For instance, plasma may influence most other body fluids, such as urine, cerebrospinal fluid, and tears. It is important to analyze the proteomes of various body fluids in the context of plasma. Theoretically, the difference between any two body fluid proteomes can reflect the function of that part of the body between them. Particularly for the kidney, there were two obviously different input and output proteomes. The kidney protein handling function can be studied by comparing plasma and urine proteome.

Though proteomics has been improving rapidly, it is probably still far from being capable of exhaustively identifying all proteins in plasma and urine. Here, the comparison method described in this paper provides an illustration of a new approach for studying organ functions with a proteomic methodology. In the future, plasma and urine samples from one individual at the same time point can be characterized for the study of an individual's kidney function. Sex-specific proteins, presumably coming from sex-specific glands, can be identified if the male and female proteomes are profiled separately and they should be removed from the kidney output proteome for the black box study of kidney function. It would be better to compare the two proteomes using unbiased quantitative proteomic

techniques. With further development of proteomic technologies, i.e., quantitative-MS-based proteomics, top-down strategy proteomics, and antibody arrays, and improvement in the data quality, such comparisons will presumably result in more meaningful and valid conclusions. More detailed descriptions of kidney functions can be obtained by comparing two or more proteomes with more exhaustive and reliable protein information, such as complete MWs, pIs, posttranslational modifications, and quantitation. Because of its distinctive input (plasma) and output (urine), it is reasonable to predict that the kidney will be the first organ whose functions are further elucidated by proteomic methods in the near future. It can also be anticipated that there will be more applications for proteomics in organ function research.

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Chapter 19
Urinary Protein Biomarker Database: A Useful Tool for Biomarker Discovery

Chen Shao

Abstract An open-access biomarker database offers a convenient tool for researchers to acquire existing knowledge about proteins and diseases by simply querying its Web site. Biologists can use the biomarker database to assess the confidence and disease specificity of their own research results by cross-study comparison, and bioinformaticians can use it to discover new relationships between diseases and proteins by reanalyzing data via new strategies. This chapter introduces the urinary protein biomarker database, a manually curated database that aim to collect all studies of urinary protein biomarkers from published literature. In the current stage, this database includes very few disease-specific biomarker candidates that have been reported by multiple studies, reflecting current status in the field of urinary biomarker discovery. We believe that this situation will be improved with the development of technologies and accumulation of data, and a more complete and precise biomarker database will play more important role in future studies.

Keywords Database · Urinary biomarkers

19.1 Rationale for a Urinary Biomarker Database

Urine is an ideal source of biomarkers. In comparison to plasma, urine has some unique advantages that make it a suitable source for both physiological research and disease biomarker discovery. Firstly, urine can be collected continuously and noninvasively. Secondly, the urinary proteome directly reflects the condition of the urinary system. Thirdly, since the urinary proteome contains a number of plasma proteins, some changes of the plasma proteome can also be found in urine.

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Therefore, urine is not only a good source for the study of urological diseases, but can also reflect the status of the entire body [4]. There have been a considerable amount of urinary biomarker studies based on different experimental platforms for a variety of diseases, such as bladder [2] and prostate cancer [12], renal disorders [3], and cardiovascular diseases [9].

The development of proteomic technologies offers possibility of identification and quantification of all peptide/proteins in biological samples simultaneously. Currently, a single proteomic assay can identify dozens or hundreds of peptide/proteins that express differently between normal and disease conditions. However, due to limitation in patient samples and experimental resource, usually only a small proportion of these 'differentially expressed proteins' were selected for consequent validation experiments, while the rest of them were absent in any further analysis or even not reported in the published articles. The relative low throughput in the validation phase decreases the efficiency of whole biomarker discovery workflow and results in a waste of the abundant information achieved in the discovery phase. Researchers need to pay attention on this 'ignored information' if they want to make fully use of the high-throughput proteomic technologies. Collecting this information into a database is the first step for in-depth data analysis.

On the other hand, a considerable amount of the 'differentially expressed proteins' identified in a proteomic analysis does not reflect the real difference between normal and disease conditions, but are caused by some relatively random factors, such as experimental errors and variation among urine samples. Enlarging the analyzed sample size is an idea solution to eliminate these influence factors, but it costs too much experimental resources since urinary proteome has been reported to vary even among healthy individuals [8] and is affected by a number of physiological factors [7, 10]. This chapter suggests that cross-study comparison may be a much easier way to partially solve this problem. The concept is that if the same trend of abundance change is observed for a protein in more than one distinct study, the chance that this observation is caused by random factors would be significantly decreased. The comparison can be highly simplified by collecting results of existing urinary biomarker studies into an open-access database.

Additionally, building a biomarker database can facilitate the assessment of disease specificity for biomarker candidates. Only biomarkers with rigorous disease specificity can be used to distinguish diseases with similar signs and symptoms and consequently guide the choice of drugs and treatments. By querying a biomarker database, biomarker candidates that are related to multiple diseases can be easily picked out, so that researchers can better focus on biomarker candidates with high disease specificity and save their efforts from those that have low potential to distinguish different diseases.

In summary, a urinary biomarker database offers a convenient tool for cross-study comparison. It can help biologists to assess the confidence and disease specificity of their own research results and allows bioinformaticians to discover new relationships between diseases and proteins by reanalyzing existing data via new strategies. A list of existing databases of normal urinary proteome or urinary biomarkers is shown in Table 19.1.

Table 19.1 Urinary proteome databases

	URL	Content
HKUPP database	http://www.hkupp.org/	Proteome of normal kidney and urine
Urinary exosomes protein database	http://dir.nhlbi.nih.gov/papers/lkem/exosome/index.htm	304 proteins identified from exosomes in normal human urine [11]
MAPU urine dataset	http://www.mapuproteome.com	1,543 normal human urinary proteins identified by Adachi et al. [1]
Clinical urine proteomic database	http://alexkentsis.net/urineproteomics/	Urinary proteins that were annotated to be associated with diseases by machine learning and text mining methods [6]
Urinary peptide biomarker database	http://mosaiques-diagnostics.de/diapatpcms/mosaiquescms/front_content.php?idcat=257	CE-MS results of naturally occurring human urinary peptides in different pathophysiological conditions [14]
Urinary protein biomarker database	http://122.70.220.102/biomarker/	A literature-curated database of protein biomarker or biomarker candidates in human and animal urine [13]

19.2 Establishment of the Urinary Protein Biomarker Database

In 2011, we published the Urinary Protein Biomarker Database (UPB database), a manually curated database compiling results of urinary protein biomarker studies from published literature of proteomic studies as well as small-scale experiments such as ELISA and Western blot. Manually, curation ensures the minimum mistakes appear in the process of database establishment.

All of the protein/peptides that were reported to have abundance change under disease conditions are considered as biomarker candidates and collected in this database. No extra filtration of biomarker confidence is used since the aim of building this database was to preserve the original result of literature and make the database as comprehensive as possible. Users can use information such as detection method and sample size displayed in the database to help assess data confidence themselves. Table 19.2 shows data statistics of this database.

Particularly, a very small proportion of records in this database are 'negative records,' in which changes of protein abundance were reported as not statistically significant under disease conditions. These records are included because the same proteins were identified as biomarker candidates for the same diseases by other studies. Including negative data in the biomarker database is important, since it may help researchers to assess data confidence better by analyzing conflict results for the same biomarker candidate.

Table 19.2 Data statistics of the UPB database after an update in July 2013

	Human dataset	Animal dataset			
		Rat	Mouse	Others	Total
Articles	348	49	19	8	76
Records	993	299	62	18	379
Diseases	119	29	13	7	49
Biomarkers	819	253	62	18	333
Proteins	458	161	62	16	239

DATABASE ID : h643

DISEASE INFORMATION :

Disease:	early IgA nephropathy
Samples:	seven healthy volunteers (without hematuria), five patients with early IgAN, and seven patients with TBMN
Tissue:	urinary exosomes
Pubmed ID:	21595033
Year:	2011

BIOMARKER INFORMATION :

Protein name:	alpha-1-antitrypsin
UniProt ID:	P01009
IPI ID:	IPI00553177
Fragments or variants:	
Abundance change:	upregulation in the IgAN group
MW(detected):	
pI(detedcted):	
PTM:	
Detection methods:	nano-UPLC MS/MS
Validation on distinct samples:	were validated on six healthy volunteers, 12 IgAN patients,and 12 TBMN patients by Western blot analysis
Additional information:	a-1-Antitrypsin in the normal group was lower than in the IgAN group and was similar to the TBMN group
Plasma protein:	yes

OTHER RECORDS OF THIS PROTEIN:

ID:	h1011	Disease:	bladder cancer	Link
ID:	h957	Disease:	IgA nephropathy	Link
ID:	h915	Disease:	idiopathic focal segmental glomerulosclerosis (FSGS) following kidney transplantation	Link
ID:	h732	Disease:	Nonmuscle invasive bladder cancer	Link
ID:	h729	Disease:	Nonmuscle invasive bladder cancer	Link
ID:	h717	Disease:	Diabetic nephropathy(type 2 diabetes)	Link
ID:	h697	Disease:	bladder cancer	Link
ID:	h564	Disease:	Systemic juvenile idiopathic arthritis	Link
ID:	h563	Disease:	Systemic juvenile idiopathic arthritis	Link

Fig. 19.1 A Webpage displaying records in the UPB database

For each biomarker candidate in this database, the following information was collected (if available): definition and sample size of the disease and control groups, experimental procedures and instrument types, protein information such as fold change, fragment, variant and post-translational modification (PTM), experimental molecular weight and pI. In particular, proteins were queried in the plasma proteome list [5] to infer its origin (Fig. 19.1).

This database is open access to nonprofit researchers in the community. The Web site now allows users to browse and download the complete database. Users are strongly welcome to submit their own data to this database.

19.3 Analyzing Data in the UPB Database

Analyzing data in the UPB database reveals some important aspects in urinary biomarker discovery and database construction. In this database, biomarker candidates identified by different proteomic methods overlapped poorly with each other. Approximately, half of the records were identified only by proteomic methods and reported in only one study. Besides false positive results generated from technic errors or limited sample size, this phenomenon might be caused by several other reasons. The first one is that authors do not always report the whole protein list that is identified to have significant abundance change in disease conditions. Sometimes, they only report proteins that are thought to have higher potential to act as biomarkers or those have not been reported by other studies. Lack of original experimental data is an instinct problem in the construction of a literature-based database. Secondly, since different proteomic strategies vary a lot in the methods of sample preparation, separation, identification, and quantification, proteins or peptides with particular property (i.e., hydrophobicity) may be preferred in one strategy but cannot be identified in another one. Thirdly, although some studies are for the same disease, their results may not be comparable due to different criteria for the selection of samples to disease and control groups. So, detailed description of patients in each group should be included in biomarker database. The poor overlap rate among different proteomic studies for the same disease in this database makes researchers difficult to perform some in-depth bioinformatical or statistical methods, such as meta-analysis.

Studies based on animal models also overlapped poorly with those based on human samples. However, considering that the inter-organism overlap rate is not lower than the overlap rate among different proteomic studies of human samples, no clear conclusion can be made to the question that how well these animal models mimic real human diseases.

In the current stage, the UPB database includes very few disease-specific biomarker candidates that have been reported by multiple studies. Whereas a large proportion of biomarker candidates in this database are considered to have relatively low potential for clinical usage due to lack of disease specificity or further validation to prove their confidence. This reflects current status in the field of

urinary biomarker discovery. We believe that this situation will be improved with the development of technologies and accumulation of data, and a more complete and precise biomarker database will play more important role in future studies.

19.4 Potential Usage of the Biomarker Database

A biomarker database facilitates researchers acquire existing knowledge about proteins and diseases. By simply querying the biomarker database, researchers can find answers to some important questions to help them assess biomarker candidates they identified. For example, they may want to know whether these proteins have been reported to be biomarkers or biomarker candidates for the same disease by other groups, and if so, whether the fold changes they observed agree or conflict with previous studies. By querying a biomarker database of animal models, they can also easily find out that whether orthologous of these proteins have been studied by animal models but still lack of validation in human samples. In addition, it is also important to know whether these proteins have been previously identified as biomarkers or biomarker candidates for other diseases, which would indicate their poor disease specificity.

A biomarker database is also a useful bioinformatics tool to study the pathophysiology of diseases with the hypothesis that diseases sharing biomarkers may share the same injury sites or pathophysiological processes. For a 'new' disease where the pathogenesis or injury sites are not clear (for example, a new drug with unknown toxicity), if the fold changes of urinary proteins caused by this disease are known, researchers can query the protein list in the database to link the disease to other diseases that cause similar fold changes in these proteins. The injury site, pathophysiological process, and severity of the 'new' disease can then be inferred by its relationship to the other diseases.

Disease–protein network is plotted to display relationships between diseases and proteins deposited in a biomarker database. Moreover, a disease–disease network can be plotted by linking diseases sharing the same proteins as biomarkers or biomarker candidates, while a protein–protein network can be plotted by linking proteins that were found to be related to the same disease. Researchers can possibly dig out novel relationships among those diseases and proteins by analyzing structures or topological characters of these networks. In the previously published article [13], we built a weighted disease–disease network in which the weight of each link was defined as the number of biomarker candidates shared by two diseases. This network was then clustered into seven densely connected subnetworks solely based on its topological structure. Most diseases in the same subnetwork are known to share similar injury sites or pathophysiological processes, indicating that the result of clustering was very rational biologically. This example suggests that network analysis of the biomarker database offers a new angle of view for the similarity among diseases, and therefore, it may be helpful to study the pathophysiology of diseases.

19.5 Future Work

Existing biomarker database only includes basic information of diseases and proteins. Extending more information about proteins and diseases to the current database can make the database more convenient for users. As listed in Fig. 19.2, the UPB database can be improved in several aspects.

19.5.1 Disease Information

Besides urinary protein/peptide biomarkers, nonprotein and non-urinary biomarkers are also essential to disease diagnosis. More biomarker data can be collected from datasets of genomic and metabolic studies as well as studies of other kinds of biological samples. Some open-access disease databases, such as the Online Mendelian Inheritance in Man (OMIM) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) disease database, also provide useful information, such as biomarkers, pathways, drugs, and drug targets.

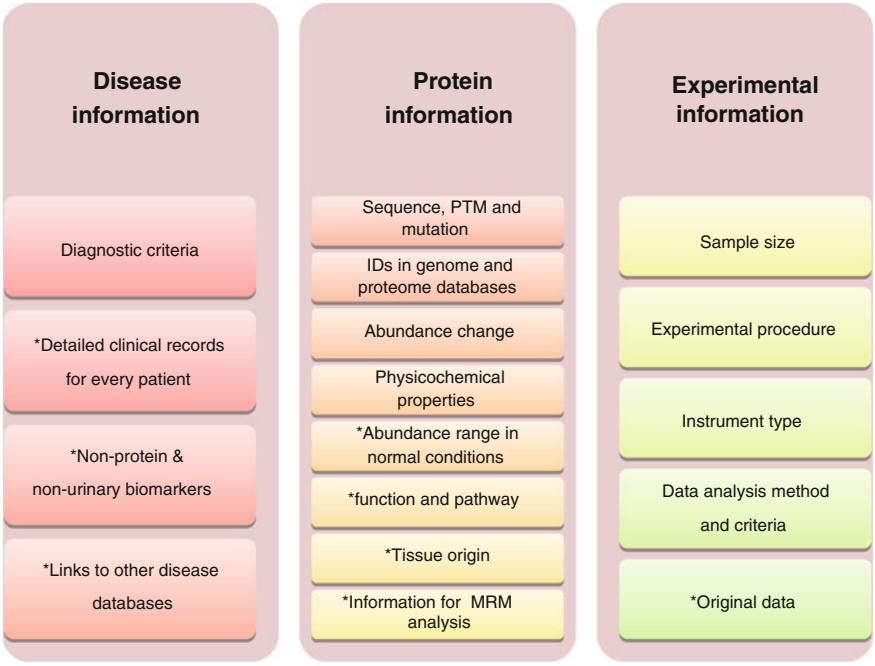

Fig. 19.2 Design for the future urinary biomarker database. Contents marked with *asterisks* have not been included in the current UPB database

19.5.2 Protein Information

Variation of urinary proteome appears in different physiological conditions and among different individuals, affecting the result of urinary biomarker study. By including abundance ranges of proteins in normal conditions, the database can offer a good reference to help researchers determining changes of protein abundance that are caused by diseases. The database can also be improved by adding more protein functional information. The functional information could be acquired from open-access bioinformatics resources, e.g., the Gene Ontology database, KEGG pathway database, protein–protein interaction databases, and the Web site of Protein Atlas Project for protein tissue expression profiles.

In addition, targeted proteomic approaches such as multiple reaction monitoring (MRM) can validate biomarker candidates in the high throughput and high-accuracy manner. To facilitate researchers whom may want to validate biomarker candidates in the database via MRM assay, list of proteotypic peptides and their MS/MS spectra for each protein can be included in the database.

19.5.3 Experimental Information

Achieving original experimental data allows database builders or bioinformaticians to reanalyze results from different studies in the same criteria, so that they can get much better control of data confidence and achieve more precise result in the cross-study comparison. However, it is hard to acquire original data in a literature-based database. Fortunately, more and more researchers would like to upload the original files of proteomic experiments to an online repository in the recent years, this situation will be improved in the near future.

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