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.

Y. Gao (✉)
National Key Laboratory of Medical Molecular Biology, Department of Pathophysiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences/School of Basic Medicine, 5 Dong Dan San Tiao, Beijing, China
e-mail: gaoyouhe@pumc.edu.cn

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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

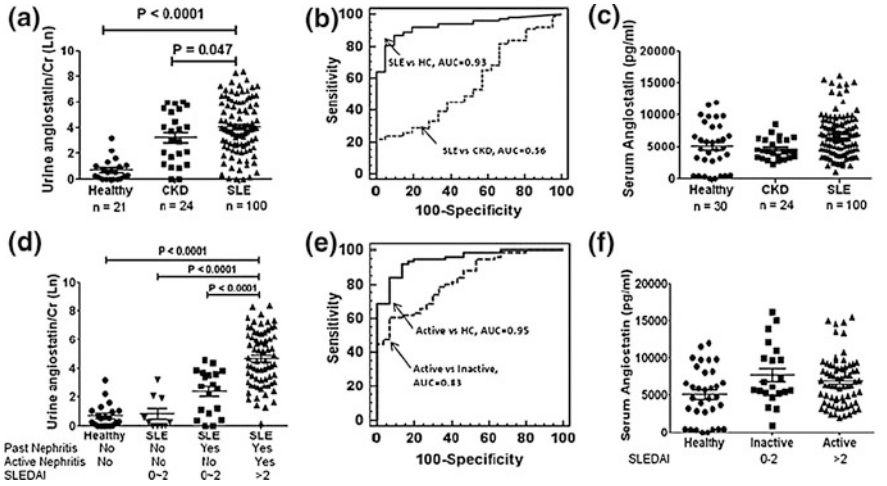

Fig. 1.1 Validation of urinary angiostatin as a marker in a larger independent cohort of SLE patients ($n = 100$), chronic kidney disease (*CKD*) patients ($n = 24$), and healthy controls ($n = 21$). **a** Urinary angiostatin levels as determined by ELISA are expressed as the natural logarithm of the 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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