In recent years, there has been an explosion of research directed at the identification and characterization of biomarkers of health and disease, driven to a great extent by advances in proteomic analysis and other technologies. Since urine sampling is relatively non-invasive and accessible, and since urine contains substances derived primarily from tubular secretion, kidney disease has been a major focus of biomarker research. But what really is a biomarker? Strictly speaking, a biomarker should be indicative of a biological process, and if it is to be adopted for clinical use, it should satisfy three criteria, as recommended by the Institute of Medicine of the National Academies of Science [1]. First, the biomarker must have analytic validity, in that testing should be reliable and reproducible across laboratories and clinical settings, and with sufficient sensitivity and specificity for the condition under consideration. Second, the biomarker must undergo qualification, with a determination that it is associated with the disease and that interventions targeting the biomarker can impact hard clinical endpoints. Finally, the biomarker must be evaluated for its utilization: in order to consider the use of the biomarker as a surrogate endpoint for the condition under consideration, it must be shown that interventions targeting the biomarker can impact hard clinical endpoints.

Keywords: angiotensinogen; biomarker; urine

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in a disease process, evidence should be particularly robust, and this usually mandates the conduct of large randomized clinical trials. In this issue of *Nephrology Dialysis Transplantation*, Mills et al. [2] add to the growing evidence that angiotensinogen, a key component of the renin–angiotensin system (RAS) and the only known substrate for renin, is a potential urinary biomarker that identifies humans at risk for chronic kidney disease (CKD).

The biology of intrarenal angiotensinogen is highly intriguing. In this respect, data from animal models have been essential to guide interpretation of human urinary studies. Angiotensinogen is synthesized primarily in cells of the proximal tubule and is secreted from the apical surface into the lumen, where it is converted first to angiotensin (Ang) I and then Ang II by tubular renin and angiotensin-converting enzyme (ACE), respectively. Indeed, the kidney contains all components of the RAS, and intrarenal Ang II formation can occur via several pathways, leading to interstitial concentrations of Ang II that far exceed circulating levels, particularly in the vicinity of the proximal tubule [3]. Circulating Ang II is also taken up by cells via an AT$_1$ receptor-dependent mechanism, increasing kidney content. After angiotensinogen enters the proximal tubular lumen, it may spillover to distal nephron segments. In the collecting duct, luminal angiotensinogen can be converted to Ang I and Ang II by local renin and ACE, before appearing in the final urine.

The activation of the intrarenal RAS contributes significantly to hypertension and CKD progression, via inappropriate elevation of Ang II levels and stimulation of kidney AT$_1$ receptors. The activation of AT$_1$ receptors is associated with enhanced intrarenal vasoconstriction and sodium and water retention, as well as adverse non-hemodynamic effects that culminate in tubulointerstitial fibrosis and glomerulosclerosis. The role of proximal tubular angiotensinogen in these pathways appears to be paramount. In support of this role, intrarenal expression of angiotensinogen substantially increases in animal models of hypertension and CKD [4, 5]. In a series of elegant experiments performed by Chan and colleagues [6, 7], transgenic mice with overexpression of proximal tubular angiotensinogen develop hypertension, albuminuria and tubulointerstitial injury and are more susceptible to diabetes-associated tubular cell apoptosis. Adverse renal endpoints in these mice are prevented by the blockade of the RAS, or by the inhibition of reactive oxygen species generation, which occurs independent of blood pressure lowering [7–9]. Thus, these studies support a vital role for intrarenal angiotensinogen-stimulated production of Ang II in the pathogenesis of progressive renal injury.

In order to put the brakes on proximal tubular angiotensinogen synthesis in CKD, it would therefore seem important to understand how the system is regulated, and here the story becomes even more interesting. In rats chronically infused with Ang II, proximal tubular expression of angiotensinogen paradoxically ‘increases’, as does collecting duct renin activity [10]. As a result, angiotensinogen spillover into the distal nephron occurs, with increased urinary excretion. This phenomenon, referred to by Navar and colleagues [10, 11] as intrarenal angiotensinogen ‘augmentation’, appears to occur as a direct consequence of Ang II–mediated AT$_1$ receptor activation in tubular cells. Indeed, rats infused with Ang II generate increased tubular Ang II in a feed-forward fashion, which serves to exacerbate hypertension and renal injury [11].

Urinary angiotensinogen levels directly reflect intrarenal RAS activity, supporting its candidacy as a biomarker. Thus, the amount of urinary angiotensinogen derived from increased glomerular permeability, as occurs in the setting of generalized proteinuria, seems quite limited [10]. Not surprisingly, with the recent development of a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for human angiotensinogen [12], the effect of hypertension or kidney disorders on urinary angiotensinogen levels has been the subject of several investigations. In a cross-sectional study involving 70 hypertensive and 36 normotensive subjects, Kobori et al. [13] showed that urinary angiotensinogen levels were positively correlated with systolic and diastolic blood pressures. In hypertensive subjects, RAS blockade attenuated the increase in urinary angiotensinogen. Increased urinary excretion of angiotensinogen has also been reported in humans with CKD due to diabetic nephropathy [14], membranous nephropathy [14], renal transplantation [15], chronic glomerulonephritis from a variety of causes [16, 17] and IgA nephritis [18, 19]. In the case of IgA nephritis, urinary angiotensinogen levels correlate with renal tissue angiotensinogen gene expression [18, 19], supporting a link to activation of the intrarenal RAS. Although these reports have been highly informative, patient numbers have been small, CKD studies were performed largely in Asian subjects and adjustment for multiple potential confounding variables has not been consistent. Thus, the applicability of these initial observations to other populations with CKD is unclear.

This brings us to the manuscript by Mills et al. [2] in the current issue. In this cross-sectional study of adult subjects, the association between urinary angiotensinogen levels (measured by ELISA on 24 h urine specimens) and the risk of CKD was determined. The study subjects were from the US population, with almost equal numbers of African Americans and Caucasians, and included a group with CKD (49% diabetic), compared with controls without CKD (n = 201 in each group). The authors report a significant increase in urinary angiotensinogen excretion in subjects with CKD (with or without correction for urinary creatinine excretion) and a significant inverse correlation between urinary angiotensinogen and estimated glomerular filtration rate (eGFR). Furthermore, although urinary angiotensinogen excretion was associated with increased albuminuria, sensitivity analysis determined that the association of urinary angiotensinogen with reduced eGFR and increased risk of CKD was independent of urinary albumin. Thus, it is unlikely that the presence of urinary angiotensinogen is accounted for by leakage across the glomerular filtration barrier in proteinuric states, as discussed above. It is somewhat puzzling therefore that plasma angiotensinogen levels were also significantly associated with increased odds of CKD in this study after adjusting for multiple variables, including...
systolic blood pressure. Of perhaps more interest, the association between urinary angiotensinogen excretion and risk and severity of CKD was independent from the use of ACE inhibitors or angiotensin receptor blockers (ARBs), a point we will revisit later.

The authors are to be commended for several aspects of the study. First, this represents the largest study in humans to address urinary angiotensinogen as a marker of CKD risk, and therefore, the results have a substantial statistical power. Second, the authors have examined the impact of multiple covariates in this study, with the use of continuous variables where possible (including the effect of plasma glucose), and the analytical methods are rigorous. The association of urinary angiotensinogen with CKD persisted after adjustment for potential confounders and is indeed quite strong (odds ratio ≥6), which was the first criterion put forward by Bradford-Hill almost half a century ago to establish causality [20]. Third, despite this being a case–control study, the authors took care to minimize bias. The control sample was chosen from the same population to minimize sampling bias, and moreover, the ‘exposure’, urinary angiotensinogen, was an objectively measured variable and not subject to recall bias, a well-recognized weakness of case–control studies.

Other aspects required to prove causality are worthy of examination. The Mills manuscript adds a measure of ‘consistency’, since the association between CKD and urinary angiotensinogen has now been reported in multiple studies, and in different populations, albeit of small size. These studies have been cross-sectional, with case–control designs, which is understandable given the lower expense compared with randomized trials, and their overall exploratory nature. However, cross-sectional studies are susceptible to selection bias; in the present study, for example, patients with a more rapid course of disease to kidney failure might have been underrepresented, and slow- or non-progressors would tend to be over-sampled, generating a biased result. Moreover, the criterion of a ‘temporal relationship’ to establish causality cannot be answered by a cross-sectional study; only a longitudinal study can truly differentiate the causative role of urinary angiotensinogen from an epiphénomène. Although ‘biological plausibility’ has certainly been established, as outlined earlier, additional ‘coherence’, especially with regard to the effect of RAS blockade on urinary angiotensinogen, would appear to be necessary. Of course, ‘experimentation’ is the last, but far from the least important step, which will prove if urinary angiotensinogen can deliver in improving clinical care.

Naturally, other questions are raised by the study results. For example, although there is undoubtedly a strong association between urinary angiotensinogen and CKD risk, the data were obtained from 24 h urine collections, which are cumbersome for patients to perform. To advance the utility of this assay as a predictor of CKD risk, it would be useful to validate the results in spot urine samples, corrected for urinary creatinine. In addition, as the authors point out, dietary sodium intake was not recorded in this study, and this may represent an important modifier of the intrarenal RAS that could impact the production of angiotensinogen. In spontaneously hypertensive rats on a high-salt diet, plasma Ang II levels unexpectedly increase by 3- to 4-fold, and urinary angiotensinogen increases 10-fold (via the augmentation phenomenon), associated with accelerated renal injury [21]. A high-salt diet in normal rats stimulates renal oxidative stress and synergizes with the effects of Ang II infusion to increase blood pressure, proteinuria and urinary angiotensinogen [22]. This process may also occur in humans with CKD. Thus, in patients with IgA nephritis, Konishi et al. [23] demonstrated that urinary angiotensinogen levels were significantly augmented upon consumption of an ordinary salt diet (12 g/day NaCl), compared with a low salt diet (5 g/day NaCl). In contrast, urinary angiotensinogen levels were not affected by changes in sodium intake in subjects with normal renal function. These observations suggest that patients with CKD may be subject to inappropriate augmentation of intrarenal angiotensinogen production by dietary salt that could accelerate disease progression.

Finally, what are we to conclude regarding the absence of association between urinary angiotensinogen and the use of ACE inhibitors or ARBs in this study? This unexpected result might be the result of bias, since cases (patients with CKD) would be more likely to be taking ACE inhibitors and/or ARBs than controls and might also be more likely to have higher urinary angiotensinogen levels at baseline, before the introduction of RAS blockade. Accordingly, without a larger longitudinal study, it is difficult to tease out the differential effect of ACE inhibitors and/or ARBs on urinary angiotensinogen. Measurement of urinary angiotensinogen in stored samples from completed large randomized controlled CKD trials involving the use of ACE inhibitors and/or ARBs could provide a swift and efficient method to address this issue. It is of interest that in animal studies, intrarenal angiotensinogen augmentation is inhibited by AT1 receptor antagonism, and small studies in humans with CKD or hypertension demonstrate decreases in urinary angiotensinogen with RAS blockade [13, 14, 18]. In the larger study by Mills et al., information on dosing of ACE inhibitors and ARBs was not reported, and medication adherence was not assessed. It is certainly possible that conventional doses of RAS-blocking agents may be insufficient to decrease urinary angiotensinogen in the face of the augmentation effect. On the other hand, if intrarenal angiotensinogen production is truly not altered by RAS-blocking agents in humans, there is a significant cause for concern. In this regard, a recent post hoc analysis of the first and second Ramipril Efficacy in Nephropathy (REIN) trials showed that a high-salt diet in humans with non-diabetic CKD was associated with an increased risk of disease progression, which was independent of blood pressure and mediated by a blunted antiproteinuric effect of ACE inhibition in these patients [24]. Whether this reflects a blunted inhibitory effect on renal angiotensinogen synthesis is unknown. Clearly, research efforts are required to improve our understanding of the regulation of proximal tubular angiotensinogen production and to specifically target the intrarenal RAS without inducing adverse effects associated with pronounced systemic (e.g. dual) RAS blockade.
The promise of a novel biomarker is that it can be used not only as a prognostic tool, but also as a surrogate outcome to guide therapy, or it may even be a therapeutic target by itself. Is urinary angiotensinogen ready for prime time as a biomarker of CKD? Not quite. Although there is support for its analytical validity, there is research to be done regarding its qualification, specifically focused on demonstrating the impact of targeting on renal outcomes. Moreover, utilization will require the conduct of clinical trials. Nonetheless, the study by Mills et al. represents a leap forward in this exciting field and holds considerable promise to someday positively impact the care of patients with CKD.

Conflict of interest statement. None declared.


References


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