

Finding New Sea Legs for Urine Proteomics

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We draw attention to a commentary published in this issue of *JASN* by Knepper¹ regarding studies undertaken in search of new biomarkers using urinary proteomics. Many of us who review or read these studies want important information to grace our journal pages. Proteomic technology today, even as it rapidly evolves, is capable of clever *measurement and presentation*, but harnessing this technology to better understand clinical or experimental disease is where true progress lies. By and large, we are not there yet. Reasonable people acknowledge this concern is more of an observation than a complaint about a discipline in its adolescence.

Identification of protein/fragment signatures in urine needs context and credible evidence of improving on what is already known. A useful experimental design should test protein signatures for relevance to pathophysiology and specificity for certain diseases. We cannot get to this level without everyone making an effort to raise the bar.

As editors of your journal, like any well-regarded journal, we are always torn between publishing new preliminary data of potential interest *versus* a more penetrating story that truly advances the ball. We hope Knepper's insightful discussion now, and what will come from new work by our community of investigators, forges greater expectations for future studies: Expectations for replication in parallel populations accompanying the initial identification of a signature, disease specificity controls where appropriate, and additional biochemical or immunologic confirmation where available. When contemplating a more advanced validation study, the design should develop along the lines of a comparative clinical trial against other markers, other diseases, or various treatments with appropriate attention to sensitivity and specificity of the results.

JASN editors going forward will expect future manuscripts using urinary proteomics to contain the above en-

hancements. If we all promote a rigorous scientific body of work, then we can be optimistic that urinary proteomics will provide a powerful tool by which we can identify new markers, understand their use, and better care for our patients.

DISCLOSURES

None.

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See related Occasional Observation, "Common Sense Approaches to Urinary Biomarker Study Design," on pages 1175–1178.

Vascular Calcification: The Three-Hit Model

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Vascular calcification, primarily arterial calcification, is one of multiple forms of extraskeletal calcification that is highly prevalent in patients with chronic kidney disease (CKD), especially those on dialysis. Arterial calcification is not a new or novel phenomenon: Mummified bodies from the ice age and many older adults and patients with diabetes and without CKD have at least some arterial calcification.¹ The presence and magnitude of arterial calcification are associated with an increase in cardiovascular events or death in the general population² and, in some studies, in dialysis patients.^{3,4} Cross-sectional and longitudinal studies in dialysis patients identify a plethora of risk factors, including advanced age, diabetes, duration of dialysis or magnitude of CKD, hyperphosphatemia, inflammation, and excess calcium-containing phosphate binders, but these associations are not consistently

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found in all studies.¹ Importantly, some patients with CKD do not have arterial calcification despite significant exposure to various risk factors, and these same patients remain calcification-free over time on dialysis. This observation suggests some individuals are protected from the devastating effects of calcification and has led to a search for protective factors or inhibitors of calcification. This concept also holds true for other forms of mineralization, because human and animal studies have demonstrated that cartilage mineralization⁵ and kidney stone growth would proceed unabated if it were not for the effects of inhibitors. Thus, much evidence supports the notion that regulation of both desirable and undesirable calcification/mineralization is at least partly mediated through the function and availability of inhibitors.⁶

Multiple inhibitors of vascular calcification are known. Local tissue inhibitors include matrix gla protein, pyrophosphates, and osteopontin, which inhibit arterial mineralization in both animal models and *in vitro* cultures of vascular smooth muscle cells.^{1,6} Fetuin-A ($\alpha 2$ Heremans Schmidt glycoprotein [Ahsg]) is another inhibitor of calcification but acts as a circulating inhibitor. It is synthesized predominately in the liver and is abundant in serum at levels of 0.5 to 1.0 g/L. The transcription and synthesis of fetuin-A are downregulated during inflammation; therefore, fetuin-A represent a reverse acute-phase reactant.⁷ Low levels of fetuin-A in patients with CKD are associated with arterial calcification, calciphylaxis, and mortality.^{8–10} Fetuin-A binds to both calcium and phosphate in the serum, forming small “calcioparticles” that are presumably removed through the reticuloendothelial system¹¹; therefore, fetuin-A acts in host defense to clean the blood of unwanted calcium and phosphate.

Targeted disruption of fetuin-A (Ahsg) in mice leads to diffuse and profound soft tissue calcification on a normal diet but only on a DBA/2 background, which is inherently prone to calcification. On the C57BL/6 background, the calcification requires very high dietary calcium, phosphorus, and vitamin D, leading to calcification in soft tissue and small arterioles of muscle, kidney, and lung.¹⁰ Additional studies demonstrated that the addition of CKD results in marked hyperphosphatemia in C57BL/6 Ahsg^{-/-} null mice, leading to myocardial and pulmonary calcification but not arterial deposits.¹² These studies suggested that fetuin-A protects against calcification in settings of genetic predisposition and CKD but that different combinations of factors lead to variable phenotype.

In this issue of *JASN*, Westenfeld *et al.*¹³ add to our understanding of calcification in CKD and the importance of fetuin-A. They hypothesize that endothelial damage is required for development of large artery calcification. To test this hypothesis, they crossed Ahsg^{-/-} null mice with ApoE^{-/-} null mice, the latter known to have increased cholesterol and atherosclerosis. They then examined whether a high-phosphate diet alone or high-phosphate diet plus CKD induces arterial calcification. All mice developed hyperphosphatemia on the high-phosphate diet, but only animals with CKD developed hyperparathyroidism. The double-deficient Ahsg^{-/-}/

ApoE^{-/-} null mice developed a similar degree of atherosclerosis as the ApoE^{-/-} mice, which is unaffected by either high-phosphate diet or CKD. In the ApoE^{-/-} null mice, high-phosphate diet and CKD led to an increase in aortic but not coronary artery calcification. In contrast, the double-deficient Ahsg^{-/-}/ApoE^{-/-} null mice developed both aortic and coronary artery calcification with high-phosphate diet alone, but this was aggravated further in the presence of CKD. Thus, extensive and multisite arterial calcification in this animal model requires genetic predisposition to atherosclerosis (apolipoprotein E deficiency), a genetic defect in an inhibitor of mineralization (Ahsg deficiency), and hyperphosphatemia that is further accelerated by CKD: A three-hit model.

Unfortunately, it seems that patients on dialysis frequently have two hits: The first hit is diabetes or hypertension with arterial tissue damage, endothelial dysfunction, and atherosclerosis, and the second hit is a disorder of mineral metabolism in the form of hyperphosphatemia and/or hyperparathyroidism. The difference in patients who do and do not develop calcification despite these similar risk factors may be the third hit. Those who have inflammation with resulting decreases in fetuin-A (or deficiency in another inhibitor) develop calcification, and those with normal inhibitors do not. Furthermore, it may not be the absolute level of fetuin-A or other inhibitor but rather the relative amount for a given level of calcium and phosphate that is important. Ideally, one could imagine characterizing the required inhibitor/phosphate ratio to predict those who develop calcification. Perhaps the future holds such a personalized medicine approach: Individualized, anti-calcification profiling leading to an ability to target high-risk patients.

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See related article, "Fetuin-A Protects against Atherosclerotic Calcification in CKD," on pages 1264–1274.

Illuminating Mitochondrial Function and Dysfunction using Multiphoton Technology

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Studying differential cell function or dysfunction within the kidney is made difficult by cellular heterogeneity, alterations in

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regional blood flow, oxygen tension, and interstitial tonicity, resulting in extremely complex anatomic and physiologic arrangements. Creative investigators have developed techniques to reduce this heterogeneity but often with loss or alteration of three-dimensional cellular associations, anatomic and physiologic cell-to-cell interactions, and harsh isolation and experimental conditions. These limitations have made “absolutes” difficult to determine, especially in relation to pathologic processes such as cell type involvement in renal ischemia. In this issue of *JASN*, Hall *et al.*¹ use multiphoton microscopy of kidney slices to compare mitochondrial parameters and differential cellular responses to stress. This approach allows them to quantify several key parameters of mitochondrial function and dysfunction in proximal tubular (PT) cells and directly compare different PT segments with each other and with distal tubular (DT) cells of the thick ascending limb (TAL). Because mitochondrial alterations play a central role in normal cell function and in response to injury, the article adds to our previous knowledge about an important area.

To understand the significance and limitations of their contribution, one must first understand what is known about differences between PT and TAL cells. Abundant mitochondria in cortical and outer medullary renal epithelial cells are necessary to meet the ATP demands of sodium transport by high-capacity aerobic metabolism. They compose 33% of the volume of proximal convoluted tubular S1 cells, 39% of the cells in the S2 segment, and 22% of the volume of cells in the proximal straight S3 segment; in the medullary and cortical TAL cells, mitochondria account for 30 to 44% of cell volume.² Differential tubular cell metabolism is also notable for the absence of aerobic and anaerobic glycolysis in the proximal convoluted tubule (S1),^{3,4} which removes an important mechanism for preserving cell ATP and viability during injury.⁵ In contrast, DT segments, including the TAL, have well-developed glycolytic pathways.^{3,4,6,7} Glycolysis occurs in the S3 segment of the proximal tubule, albeit to a lesser extent than in distal tubules, and contributes to maintaining ATP there when mitochondrial function is impaired.^{3,8}

Mitochondrial ATP production depends on substrate-supported, electron transport-mediated proton extrusion from the mitochondrial matrix that generates a proton gradient across the inner mitochondrial membrane. In turn, this is used to drive phosphorylation of ADP to ATP by proton movement down the gradient back into the matrix through the inner membrane F_1F_0 -ATPase. Electron transport-driven proton extrusion is also responsible for the net potential across the inner membrane ($\Delta\Psi_m$), and $\Delta\Psi_m$ in combination with the pH gradient accounts for the net proton electrochemical gradient across the membrane, also called the proton motive force. $\Delta\Psi_m$ is the larger of the two components of proton motive force under both normal and pathologic conditions⁹; therefore, it serves as a valuable index of the state of the entire system, as shown in recent studies documenting a major role for nonesterified fatty acids in the persistent mitochondrial