markers are better reflective of acute injury of tubular cells than of chronic interstitial damage. Thus, in chronic slowly progressive diseases, one should not expect a significant increase in urinary excretion of markers reflective of severe acute tubular-cell damage. At present, no solid data are available in support of a role for tubulointerstitial biomarkers in routine clinical use for the follow-up of CKD. Tests that can measure *per se* the actual tubular function instead of acute tubular-cell damage may eventually be useful in assessment of chronic tubulointerstitial disease.

The question arises of how one can measure renal function to assess the efficacy of treatment intended to dampen the progression of renal damage. Creatinine and its derivative formulae, and creatinine clearance, are not the best markers to gauge renal function in the presence of proteinuria.[10] The use of low-molecular weight proteins, such as cystatin C, also does not add much value to assessment of renal function. Even when cystatin C and plasma creatinine together were taken into the equation, they were found to be insensitive in detecting low-level reduction in the nephron mass, particularly in patients with well-preserved renal function.[11–13]

Finally, the experience of decades indicates that the direct measure of glomerular filtration rate seems ideally suited to assess renal function. However, the routine use of exogenous substances poses a dilemma. Simplified radioisotopic methods have been proposed, which may be of real practical use in nephrology centers where a large number of patients can be seen. Thus, it seems that routine estimation of renal function can be gauged by measurement of plasma creatinine or cystatin C and their derivative formulae, but periodic checking of the true glomerular filtration rate is advisable, and the use of tubular markers to assess the progression of diabetic nephropathy and thus to influence therapeutic regimens seems premature at present.

DISCLOSURE
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Phosphate, oxidative stress, and nuclear factor-κB activation in vascular calcification

Ziyad Al-Aly[1,2]

Phosphate-induced vascular calcification, characterized by induction of osteogenic programs, mineral vesicle release, and apoptosis, is prevalent in patients with kidney disease. Zhao *et al.* provide a mechanistic link between phosphate-induced calcification and increased mitochondrial membrane potential, increased mitochondrial reactive oxygen species, activation of the nuclear factor-κB pathway, and subsequent expression of osteogenic genes and vascular mineralization. This link clarifies the intracellular mechanism of vascular calcification and may allow exploration of antioxidants as therapeutic agents for vascular calcification.

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Vascular calcification is highly prevalent in patients with kidney disease and is associated with increased risk of cardiovascular events and death.[1] Once thought to be a passive degenerative process, vascular calcification is now known to be a highly orchestrated process characterized by significant phenotypic changes and molecular reprogramming that entrains a repertoire of transcription factors and osteogenic programs.

There are multiple mechanisms for vascular calcification. Particularly relevant to vascular calcification in patients with kidney disease are the mechanisms related to increased phosphate.[2] Elevated serum

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phosphorus is associated with increased calcification scores, increased risk of cardiovascular events, and all-cause mortality. Interventions aimed at lowering serum phosphorus concentrations may reduce the risk of cardiovascular events and improve survival. Over the past decade, multiple *in vitro* and *in vivo* experimental models have been developed to study the mechanisms of phosphate-induced vascular calcification.

Increased levels of phosphate induce calcification in smooth muscle cells in a dose- and time-dependent fashion. Phosphate entry into vascular smooth muscle cells is mediated via the type III sodium-dependent phosphate cotransporters Pit-1 and Pit-2. Pit-1 seems to be expressed more abundantly in human vascular smooth muscle cells.[3] Incubation of vascular smooth muscle cells in high-phosphate medium induces phosphate uptake into the cells and increased calcification. This is an active process characterized by phenotypic changes of vascular smooth muscle cells into osteochondrogenic-like cells and the elaboration of a repertoire of osteogenic signals, including increased expression of Runx2/Cbfa1 and osteopontin. Treatment of vascular smooth muscle cells with a competitive inhibitor of the sodium-dependent phosphate cotransporter phosphonormic acid causes a dose-dependent inhibition of phosphate uptake and calcification and abrogates the osteogenic program (increased expression of Runx2/Cbfa1 and osteopontin).[3] Further experiments in which the type III sodium-dependent phosphate cotransporter (Pit-1) was knocked down showed that Pit-1 knockdown cells treated with high levels of phosphate exhibited reduced phosphate uptake, decreased levels of calcification, and decreased expression of osteogenic markers compared with control cells incubated in the same medium.[3] Furthermore, restoration of phosphate uptake in Pit-1 knockdown cells by overexpression of mouse Pit-1 rescued elevated phosphate-induced mineralization. *In vivo* studies have also supported a role of phosphorus in vascular calcification. Uremic mice fed a high-phosphorus diet show increased expression of Pit-1 and Runx2 in calcified aorta, and treatment with phosphate binders attenuated this process.

Another mechanism of phosphate-induced vascular calcification includes smooth-muscle-cell apoptosis and matrix vesicle formation. In conditions of elevated calcium and phosphate, vascular smooth muscle cells undergo apoptosis and phenotypic changes into osteo/chondrocytic-like cells releasing vesicles that contain preformed crystalline apatite crystals.[2] Phosphate-induced calcification is initiated by the release of membrane-bound matrix vesicles from living cells and apoptotic bodies from dying cells. Vesicles released by vascular smooth muscle cells after prolonged exposure to calcium and phosphate contain preformed basic calcium phosphate and calcify extensively. In *ex vivo* experiments, human arterial rings from predialysis and dialysis patients, but not from healthy control subjects, showed significant accumulation of calcium with long-term exposure to elevated calcium and/or phosphate, suggesting perhaps that the uremic environment compromises the calcification-inhibitory mechanisms in the vessel wall.[4] Calcified arterial rings from predialysis and dialysis patients had increased smooth muscle loss, increased vascular smooth muscle-derived vesicles, and increased apoptosis, and treatment with a pan-caspase inhibitor, ZVAD, ameliorated calcification.[4] Under conditions of long-term exposure to elevated calcium and phosphorus, the majority of vascular smooth muscle cells in normal vessels exhibited extensive intracellular mitochondrial damage and calcification, whereas the mitochondria of vascular smooth muscle cells in dialysis vessels were intact.[4] This provides evidence that dysregulated divalent ion homeostasis may compromise mitochondrial architectural integrity. All the above taken together suggests that disturbances in divalent ion homeostasis activate calcification mechanisms, leading to enhanced expression of osteogenic markers, increased vesicle release, apoptosis, and phenotypic changes in the vascular smooth muscle cell.

Oxidative stress generally represents the imbalance between increased production of reactive oxygen species (ROS) and the reduction in expression and/or activity of cellular antioxidant defense mechanisms (such as superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxin, and sulfiredoxin). Sources of ROS include leakage of activated oxygen from mitochondria during oxidative phosphorylation and superoxide-producing enzymes such as xanthine oxidase, NADPH oxidases, and cytochrome P450. Disturbances in the normal redox state at the cellular or tissue level can lead to damage through the production of peroxides and free radicals. At low levels, some oxidative species, however, act as messengers, a phenomenon often referred to as redox signaling.

Oxidative stress is commonly observed in patients with kidney disease. Although the association between oxidative stress and vascular calcification has been noted in clinical epidemiology for a while, evidence linking oxidative stress to activation of osteogenic programs and vascular calcification has started to emerge only relatively recently. Hydrogen peroxide (H_2O_2), which is a cell-permeable ROS (and a proinflammatory second messenger), was shown to induce osteogenic gene expression, suppress smooth muscle cells' molecular signature, and induce calcification.[5] H_2O_2-induced calcification was abrogated in Runx2 knockdown cells, indicating that the mechanism of H_2O_2-induced calcification is Runx2 dependent. H_2O_2-activated phosphatidylinositol-3′-kinase–AKT signaling, but not extracellular signal-regulated kinase or phospholipase Cγ signaling, is required for H_2O_2-induced vascular smooth muscle calcification. A recent study showed that H_2O_2 is increased and colocalizes with the osteogenic transcription factors Msx2 and Runx2 in calcified human aortic valves.[6] In these valves H_2O_2 production is the result of uncoupling of nitric oxide synthases (and not NADPH oxidase), and reduced expression/activity of cellular antioxidant mechanisms, including superoxide dismutase and catalase.

Zhao and collaborators[7] (this issue) extend the above findings and examine the effect of β-glycerophosphate (BGP)-induced calcification on mitochondrial membrane potential, mitochondrial ROS, and the nuclear factor-κB (NF-κB) pathway in bovine aortic smooth muscle cells and in adenine-fed rats. The investigators

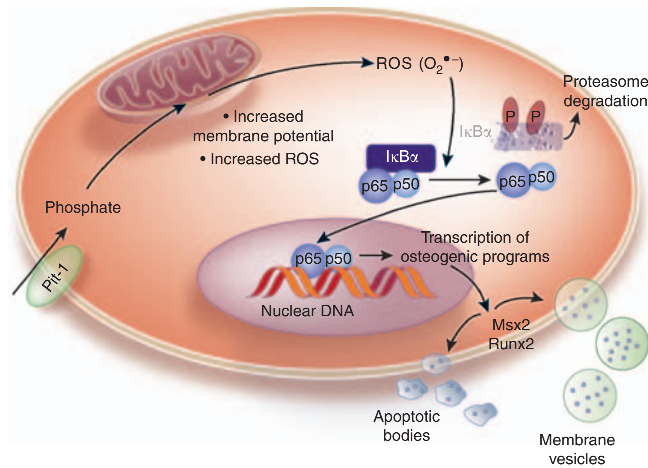

Figure 1 | Phosphate-induced vascular calcification is mediated through activation of mitochondrial reactive oxygen species, and p65 nuclear translocation. Phosphate entry into the cell occurs via Pit-1 or possibly other transport mechanisms. Phosphate stimulates mitochondrial membrane potential and leads to increased production of reactive oxygen species (ROS). Superoxide ($O_2^{\bullet-}$), an ROS, activates the nuclear factor-κB (NF-κB) pathway with IκBα phosphorylation and subsequent proteasome degradation. The activated NF-κB is then translocated into the nucleus, where it binds to specific sequences of DNA and leads to increased expression of osteogenic transcription factors. The activation of membranous ossification programs leads to phenotypic changes in the cell as well as the release of membrane vesicles and apoptotic bodies, both containing hydroxyapatite crystals.

report that BGP increased calcification by increasing mitochondrial membrane potential and consequently increasing mitochondrial ROS, specifically superoxide (O_2). They show that blockade of mitochondrial ROS abrogates BGP-induced calcification, and that it reduced the expression of the osteogenic markers Cbfa1 and Msx2. The investigators also show that BGP activates the NF-κB pathway with increased IKKβ phosphorylation, IκBα degradation, and nuclear translocation of p65 and that blockade of mitochondrial ROS abrogates that effect. Adenoviral overexpression of IκBα and knockdown of p65 reduced BGP-induced calcium deposition in these cells. The authors also examined the effect in adenine-fed rats and show that uremic rats developed increased calcium deposition, increased mitochondrial ROS levels, decreased IκBα, and increased nuclear translocation of p65, and that blockade of mitochondrial ROS by the use of MnTMPyP (Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride), a superoxide dismutase/catalase mimetic, abrogates that effect. BGP-induced calcification may not be mediated principally by ROS produced through NADPH oxidase or xanthine oxidase, as pharmacologic blockade of these pathways has no effect on calcification.

The importance of the study by Zhao and collaborators[7] is that it identifies BGP as an inducer of H_2O_2, and more specifically mitochondrial superoxide ($O_2^{\bullet-}$) (Figure 1). This novel link between BGP, a phosphate donor, and oxidative stress (both are prevalent in patients with kidney disease, and both are implicated in the pathobiology of vascular calcification) could have important clinical and therapeutic implications. The work is conceptually coherent and contributes to an enhanced understanding of the intracellular mechanism of phosphate-induced vascular calcification in cell-culture models and *in vivo*. Previous studies have linked oxidative stress and activation of the NF-κB pathway, and it has been proposed that this is probably required for increased cellular survival in response to oxidative stress-induced cell damage and death.[8] Tumor necrosis factor-α (TNF-α), a pro-osteogenic cytokine, stimulates peroxide production through NADPH oxidases and leads to increased generation of peroxide signals elaborated from mitochondrial activity and subsequently enhanced expression of membranous ossification programs (BMP-2, Msx2, Wnt3a, Wnt7a, and alkaline phosphatase in the arterial wall).[9,10] The elaboration of these membranous ossification programs (Msx2–canonical Wnt signaling cascades), which are dependent on peroxide signals elaborated from mitochondrial activity and downstream of TNF-stimulated NADPH oxidases, is also NF-κB dependent.[9,10] The convergence of both phosphate and TNF-α (at least in part) on mitochondria-generated ROS and subsequent activation of the NF-κB pathway suggest that this axis may be central to the pathobiology of calcification. It would be interesting to know whether other procalcific or pro-osteogenic moieties in patients with kidney disease also stimulate mitochondrial oxidative stress. Further studies are needed to clarify the effect of mitochondrial ROS blockade on phosphate entry into the cell (via Pit-1 or other mechanisms), mineral vesicle formation, apoptosis, and the canonical Wnt signaling pathway.

Finally, the study by Zhao *et al.*[7] expands our understanding of phosphate-induced vascular calcification; it delineates a clear link between phosphate, mitochondrial oxidative stress, and the NF-κB pathway and paves the way for future research to further elucidate the intracellular mechanisms of phosphate-induced vascular calcification. This effort may eventually lead to the exploration of antioxidants as therapeutic agents for vascular calcification.

DISCLOSURE

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How to use biomarkers efficiently in acute kidney injury

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We discuss the performance of novel biomarkers in acute kidney injury (AKI). Comparison of the areas under the receiver operating characteristic curves of several biomarkers with some clinical and/or routine biochemical outcome parameters reveals that none of the biomarkers has demonstrated a clear additional value beyond the traditional approach in clinical decision making in patients with AKI. Unscrutinized use of these biomarkers may distract from adequate clinical evaluation and carries the risk of worse instead of better patient outcome.

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"When you search in the stars for what lies before your feet you risk to stumbling over the cobblestones."
freely translated from Schopenhauer
(Source: Internet quotes)

Urinary biomarkers in the field of acute kidney injury (AKI) are a hot topic. A PubMed search using the terms 'biological marker, urine' and 'kidney injury, acute' revealed 158 papers on humans published in the past 24 months. The large majority, as well as their accompanying editorials, start or end with a statement that 'urinary biomarker X' is very promising for clinical management of AKI and detection of renal damage before a fall in glomerular filtration rate is noticeable from a rise in serum creatinine (SCr) and/or urinary output. Do the data really support this optimistic view, and can unrestricted implementation of biomarkers in clinical practice be recommended at present?

The paper by Endre *et al.*[1] in this issue of *Kidney International* does not support this view. In patients admitted to the intensive care unit (ICU), the areas under the receiver operating characteristic curves (AUCs) of biomarkers were compared for diagnosis and prediction of AKI, need of renal replacement therapy (RRT), and/or mortality. The overall performance of the six biomarkers was poor, taking into account that an AUC of 0.5 reflects the diagnostic accuracy of random allocation.

Figure 1 and Table 1 show the AUC values of different biomarkers, clinical scores, and routine biochemical parameters (RIFLE classification or Screa) as retrieved from human studies on AKI. A huge variation in the values is obvious.

What are the possible explanations of the inconsistent results obtained with these biomarkers? Some studies[2,3] show AUC values greater than 0.9 but, although truly positive, suffer from problems of generalizability, as they include homogeneous populations with a well-defined single injury to the kidney and with little or no additional comorbidity. In more heterogeneous populations such as adult cardiac surgery and, certainly, general ICU patients, the performance of biomarkers to detect AKI declines rapidly, with much lower AUC values.[1,4,5] The poor performance of urinary biomarkers in more general clinical conditions has recently been confirmed by Metzger *et al.*[6] and could be overcome only by use of a panel of 20 urinary peptides. The novelty of the paper by Endre *et al.*[1] is their attempt to evaluate whether specific biomarkers might perform better in specific clinical conditions—for example, in patients with versus without preexisting kidney disease, or with differing (presumed) timing of the kidney injury. Although this strategy indeed improved the results, enthusiasm is tempered by some more in-depth considerations. First, these new 'categorized criteria' are 'predictions of the past' and need to be validated in different populations. Experience teaches that diagnostic accuracy plummets at such validation procedures. In addition, all the biomarkers in the EARLYARF trial have been determined in nearly ideal research environments. The switch to commercial kits will further add to the diagnostic inaccuracy, along with the variability of the cutoff values of different markers as reported in many studies.[7] Second, although the stratification criteria applied by Endre *et al.*[1]—underlying kidney function and timing of insult—seem to make sense, the time to insult had to be estimated for a substantial part of the population, and in clinical practice this information is often lacking. The majority of deaths after AKI occur in patients in whom the time of the renal insult is unknown.[8] Moreover, not knowing the time of insult is most problematic in patients with smoldering disease, such as sepsis—exactly the patient population that would benefit most from a robust AKI marker. In

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