Biomarkers: more than just markers!*

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ABSTRACT

In the strive for optimal indicators of kidney damage, nephrologists may at times feel entangled in a molecular jungle. Contrary to cardiologists that succeeded in establishing highly sensitive biomarker molecules for hypoxic cellular damage, nephrologists await well-performing ‘damage’ indicators. Reasons for this are ample and mostly relate to the complex composition of the functional units along the nephrons and the diverse cellular phenotypes that may be affected by different insults (hypoxia, direct and indirect cell toxicity, autoimmunity, apoptosis and necrosis). Besides elegant studies that confirm the appropriateness of biomarkers to indicate, early and adequately, kidney damage and putatively predict outcome for kidney function, there is a need to understand the (patho-)physiological roles that these molecules play in health and disease. In this respect, a recent study by Humphreys et al. (J Clin Invest, 2013; 123: 4023) shed some light on the (patho-)physiological role that kidney injury molecule 1 (KIM-1) may play. By establishing transgenic mouse models with confined KIM-1 overexpression in proximal tubular cells, the authors are able to dissect cause and consequence, and link KIM-1 expression per se with interstitial inflammation and fibrosis. This study is remarkable for several reasons, given the profound insights into the pleiotropic functions of a single molecule, the simplicity of its design and the inclusion of adequately performed control experiment.

Keywords: acute kidney injury, chemotaxis, kidney injury molecule-1, renal fibrosis, unilateral ureteral obstruction

THE EVOLUTION OF KIDNEY BIOMARKERS

The term ‘biomarker’ is typically used for a laboratory measurement that supports the diagnosis of a disease or reflects disease activity. In extension to this definition, biomarkers for kidney diseases may also serve to improve our understanding of acute kidney injury (AKI) and even suit as potential target for AKI treatment.

Nowadays, advanced experiments are undertaken to causally link renal biomarkers with renal disease, as happened in one of the recent issues from the Journal of Clinical Investigation by Humphreys et al. [1]. However, quite some time ago it was not even clear that urine originates from the kidneys. One of the first historical sources of ‘renal medicine’ dating back to 400 B.C., refers to the achievement of Hippocrates linking bubbles on the surface of urine with kidney disease (Figure 1). More than 2000 years later, there was evidence for protein being the cause for the latter observation when Fredrik Dekkers boiled urine with acetic acid to precipitate urinary protein. At about the same time, a further cornerstone of progress in renal biomarker development was the ability to measure creatinine in blood using a picric acid-based colorimetric reaction first described by Max Jaffé. In 1931, halved urine output was described following halving of kidney mass by unilateral nephrectomy (Figure 1).

Despite recent consolidation of consensus diagnostic criteria for AKI [2, 3], in most cases early diagnosis of AKI within hours following inciting damage is, by current clinical and laboratory methods, not feasible. Serum creatinine levels and urinary output indicate AKI many hours to several
days later than decrease of glomerular filtration rate occurs. Some improvement may originate from serum cystatin C when body composition is changed, such as in children, anorectic and older adults [4]. Importantly, acute tubular damage has recently been recognized to be a major and early pathophysiological event. Technological progress using unbiased genome-wide association analysis or hypothesis-driven comparative urine analysis enabled the discovery of tubular damage markers predominantly in urine, such as kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin, interleukin 18 (IL-18) and cell cycle arrest markers. Biomarkers of acute tubular damage have been studied for early diagnosis and recovery from AKI, differentiation of pre-renal from intra-renal causes of AKI and prognosis of renal function over time [5]. Just recently, tubular damage markers have been suggested to guide management of AKI [6], a medical emergency which frequently occurs in hospitalized patients, especially in those with critical illness. Emphasizing the prognostic value of AKI even if asymptomatic—which is the majority of affected patients during the initial phase of the syndrome—the term ‘renal angina’ has been introduced [7].

**KIDNEY MARKERS OF MORE THAN ONE INJURY MODE**

The type, extent and timing of tissue damage likely determine the prognosis of kidney diseases. Ideally, one would obtain a kidney tissue specimen by biopsy to define and correlate damage with specific histological findings, which is, however, not performed due to possible complications by the procedure and the lack of sequential information over time. Thus, kidney damage ‘biomarkers’ may provide advantages over biopsies. However, the idea of a single ‘biomarker’ being suitable for different insults comes close to wishful thinking, which is exemplified by two examples with different modes of kidney injury (provided in Figure 2): a patient diagnosed with sarcoidosis by histological analysis demonstrates massive infiltrates of activated immune cells, resulting in interstitial nephritis and scarring (Figure 2A and B). The underlying cause of deteriorated kidney function and architecture is the autoimmune response with release of inflammatory cytokines that may affect different regions along the nephron. The hallmark of kidney damage in this scenario is a diffuse immune cell infiltrate. In another patient diagnosed with plasma cell dyscrasia protein casts are the cause of kidney injury with occlusion of distal tubules and collecting ducts (Figure 2C and D). As further sequelae of urinary congestion interstitial inflammatory cell numbers are also increased.

Thus, two distinct pathophysiological mechanisms cause AKI, affect specialized cells along the nephron and interstitium. It is unlikely that a single injury marker is able to indicate both mechanisms of damage to a similar extent, given differing expression patterns in interstitial, proximal and distal tubular as well as collecting duct cells. Furthermore, AKI differs from acute myocardial ischaemia/infarction by the complexity of putative damaging events. Whereas hypoxia is the predominant single cause of myocardial damage, kidney cells may be harmed by different mechanisms, at times in combination, such as toxicity (drugs), nephron congestion due to rhabdomyolysis/cast nephropathy, ischaemia/hypoxia and autoimmune disease/inflammation.

Given these considerations one should primarily differentiate between the differing causes of kidney damage, choose
the likely candidate for monitoring/diagnosing cellular tissue damage or alternatively consider the suitability of a set of biomarkers in a search towards underlying (patho-)mechanisms of AKI.

**PRELIMINARY EVIDENCE FOR AKI-TO-CHRONIC KIDNEY DISEASE TRANSITION**

After damage, the kidney has the ability to repair itself to a certain degree. With mild injury, this repair may be performed *ad integrum* and become indistinguishable from healthy tissue. However, when acute tissue damage is more severe or superimposed on pre-existing kidney impairment, the repair process may lead to (partial) organ fibrosis, which may be the first step towards chronic kidney disease (CKD). Thus, AKI may not represent a fully benign event and rather set the stage for chronic disease with structural alterations of the kidney tissue architecture [8, 9]. Evidence grows for a causal link of AKI with the *de novo* development or progression of CKD, as supported by epidemiological studies [10]. Even ‘apparently’ full kidney recovery confers an increased risk for subsequent development of CKD [11]. Also, it is known that the longer AKI persists, the greater the likelihood for chronic kidney function loss, as exemplified in the setting of kidney transplantation where increased cold ischaemia duration is accompanied by reduced function, even after correction for baseline creatinine serum values, and the number of human leucocyte antigen mismatches.

Hence, the ‘Acute Dialysis Quality Initiative’ recommends modified AKI diagnostic criteria that include tubular damage biomarkers complementary to markers of renal function. The appropriateness of renal ‘biomarkers’ in different settings (see clinical examples in Figure 2) and modification of clinical concepts on AKI during the last few years bring us to the fundamental question of what biomarkers can teach us on renal pathophysiology.

**NOVEL FINDINGS IN THE HIGHLIGHTED STUDY**

While previous experimental studies focussed on investigation of candidate genes or molecules as potentially useful ‘biomarkers’ for detecting persistent acute tubular damage, the experimental approach of a recent study by the Bonventre...
group addresses a pivotal aspect in biomarker research, that is the causal relevance of a distinguished marker protein for organ injury and inflammation. In this respect, the group focussed on kidney injury molecule (KIM)-1 [also denoted T-cell immunoglobulin and mucin domain molecule (TIM)-1, hepatitis A virus receptor-1], which has been established as the most strongly up-regulated protein in proximal tubular cells following acute kidney hypoxia. Furthermore, studies demonstrated up-regulated KIM-1 expression in CKD, such as murine polycystic kidney disease [12], with chronic tubular cell stress [13] and following ureteral obstruction [1]. Their experimental approach centred around the design of a transgenic animal strain with genetically tailored KIM-1/alkaline phosphatase (AP) expression in proximal tubular cells. The transgene was driven by constitutive Cre recombinase expression linked to the Six2 promoter [14], which was achieved to a large extent (except for occasional mosaic expression in podocytes). Immunohistochemistry revealed that a subset of 10–20% of tubules was positive for AP expression, which served as a marker protein. Notably, the authors excluded off-target effects of their transgene by determining normal phenotypes in animals with KIM-1/AP transgene expression in podocytes (Cre recombinase driven by the podocin promoter) or AP expression in Six2 Cre animals, the latter excluding direct AP toxicity. The kidneys of the bigeneric KIMRECtg strain revealed marked differences at birth, that is, a 23% lower weight, as well as 43% lower nephron number. These clues to KIM-1 playing a role in kidney development and organogenesis were unexpected and complicated the further analyses. As a consequence, the authors also tested for subtle end-organ damage in the heart that may be explained by arterial hypertension. However, normal blood pressure of transgenic animals at birth and the time course of cardiac hypertrophy argued against a pacemaker role of arterial hypertension for kidney damage in KIM-1 expressing animals. Starting 4 weeks post-partum immunohistochemistry and collagen stains demonstrated profound changes within the kidneys of the KIMRECtg strain. Focal interstitial fibrosis surrounding isolated tubules were the primary signs of an imbalance, which progressed to full-blown interstitial nephritis with massive leucocyte infiltration and ensuing tissue scarring. The KIMRECtg strain animals died at a median age of 19 weeks due to uremia, with concurrent normocytic anaemia, hyperphosphataemia and hypoalbuminaemia. Whereas no significant proteinuria was detected at birth and until 2 weeks, overt proteinuria developed from 4 weeks on. At this time point, CD3+ lymphocytes and F4/80 macrophages and dendritic cells were detected as interstitial infiltrates. Neutrophils were not abundant in the infiltrates, which contradicts an involvement of KIM-1 in regulating their influx (e.g. via receptor leucocyte mono-immunoglobulin-like receptor-5) in this transgenic animal model. Urine samples revealed proteolysis of tubular KIM-1 protein, which was detected at elevated levels at 4 weeks, as were proinflammatory cytokines and chemokines (e.g. MCP-1). In extension to this study with overexpressed KIM-1 protein in tubular cells, the authors also designed a mouse strain with mutant exon 3 of the KIM-1 gene resulting in a protein that lacks the extracellular mucin domain. Following unilateral ureteral obstruction, damage scores were determined in these animals for both kidneys revealing that mutated KIM-1 protected tubular cells and (partially) prevented renal fibrosis.

The authors also address the issue of KIM-1 orchestrating cytokine and chemokine expression in tubular cells by setting up an in vitro cell model. In porcine, proximal tubular cells KIM-1 was ectopically overexpressed, and as a result transforming growth factor-β, IL-6 as well as MCP-1 were detected in elevated concentrations in the supernatant, which resulted in up-regulated chemotaxis of differentiated primary and immortalized macrophages. The chemotaxis was abrogated by anti-MCP-1 ‘neutralizing’ antibody, suggesting that this chemokine plays a dominant role in the recruitment of the inflammatory cell infiltrate.

DISCUSSION

Since its first description as a tubular stress protein with up-regulation in proliferating and dedifferentiated cells in 1998 [15], >15 years have passed before a pivotal question of its pathophysiological relevance has been addressed in the study by Humphreys et al. [1]. The study takes advantage of cell-restricted expression of KIM-1 in tubular cells and is centred around the findings within the kidney observed over time, that is ‘spontaneously’ developing inflammation, cell infiltrates and fibrotic alterations within the tubulointerstitium. The elegance of the study resides in its straightforward design; nevertheless, some questions arise and remain unanswered. The authors do not report on systemic KIM-1 levels determined in serum samples that may be elevated in transgenes. Given the relevance of KIM-1 in T as well as B-cell biology [16–19] it remains unclear how immune cells develop in transgenic mouse strains that exhibit overexpressed KIM-1 in tubules, especially as the authors later observe cell infiltrates around vessels that resemble lymph nodes. Similar questions relate to the innate immune system, which may also be affected by KIM-1 expression [20].

Given that KIM-1 is a functional phosphatidylserine receptor and permits phagocytosis of apoptotic cells by tubular cells [21] it remains unclear, to what extent KIM-1 expression is protective for kidney damage and a rescue molecule to resolve cellular debris accumulation versus its proinflammatory role under circumstances of designed tubular overexpression. To this extent, it would be informative to test how KIM-1 itself acts as a stress protein in tubular cells and may deteriorate cell metabolism or the proteasome. Another array of questions has been put forward by the authors themselves, given that the expression of chemokines and cytokines was up-regulated in KIM-1 overexpressing cells. It will be informative to dissect the underlying regulatory events and to place KIM-1 within this regulatory network. What is upstream of KIM-1 and drives KIM-1 protein expression, what is downstream and regulated by KIM-1? Finally, one wishes to ask, whether KIM-1 is an appropriate therapeutic target, as put forward by another review [22] with the title ‘KIM-1/TIM-1: from biomarker to therapeutic target?’. The presented study suggests that excessive KIM-1 expression in tubular cells is harmful for the kidney.

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Simple translation into a therapeutic neutralization strategy may oversimplify the issue, given that KIM-1 is involved in complex cell–cell communications. The novel animal model with mutated extracellular mucin domain may, however, be an ideal starting point to test for the hypothesis of KIM-1 deleterious effects in different kidney injury models.

OUTLOOK

KIM-1 appearance and tubular overexpression are sufficient to incite tubulointerstitial nephritis and immune cell recruitment, hallmarks of chronic proteinuric kidney diseases. The study is not comparative to the point where different kidney injury markers are determined in the same transgenic disease model. In clinical settings where the timing of renal injury/tubular damage is unknown, expression of KIM-1 may be less accurate [23–26]. One of the main issues is the appropriate interpretation of elevated KIM-1 levels in the context of kidney injury and the cost-effectiveness of routine performance of such determinations.

While current biomarkers indicate tubular damage which was previously undetected, new biomarkers that are capable of uncovering the predominant type of injury (ischaemic, toxic or inflammatory) or the specific site of damage (vascular, proximal or distal tubules) need to be developed to prompt specific therapies. Future therapeutic trials should incorporate biomarkers specific to the aetiology of the AKI, and treatment should match the phase of injury.

MAIN CONCLUSIONS

(i) Progressive inflammatory kidney damage develops in a murine model with constitutive proximal tubular KIM-1 overexpression.

(ii) The extracellular mucin domain of KIM-1 mediates kidney damage, given that mice with mutant exon 3 of the KIM-1 gene lacking the mucine domain are (partially) protected from interstitial alterations with ureteral obstruction.

(iii) KIM-1 up-regulates cytokine and chemokine synthesis in tubular cells with release of chemoattractants.

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CONFLICT OF INTEREST STATEMENT

M.H. received lecture fees and reimbursement of travel costs from Abbott, Astute and Alere. These companies are involved in the development of renal biomarkers. P.R.M. declares no competing financial interests.

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