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Neutrophil gelatinase-associated lipocalin—an emerging troponin for kidney injury

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Introduction

When a subject presents with symptoms of *angina pectoris*, measurement of biomarkers such as troponin that are released from damaged myocytes can rapidly identify acute myocardial injury, allowing for timely interventions and a dramatic decrease in mortality. The analogous condition of the kidney, acute kidney injury (AKI), has been referred to as *angina renalis*, and the similarities end right there. AKI is largely asymptomatic, and establishing the diagnosis in the estimated 5% of hospitalized patients and a third of

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intensive care patients who suffer from the disease currently hinges on serial serum creatinine measurements. Unfortunately, creatinine is a notoriously delayed and unreliable indicator of AKI for a variety of reasons [1,2]. Ironically, animal studies have identified several interventions that can prevent and/or treat AKI if instituted early in the disease course, well before the serum creatinine even begins to rise. The lack of early biomarkers has crippled our ability to translate these promising findings, and human AKI remains a major risk factor for a number of non-renal complications and an independent contributor to the high mortality rate [3].

The pursuit of improved biomarkers for the early diagnosis of AKI and its outcomes is an area of intense contemporary research. For answers, we must turn to the kidney itself. Indeed, understanding the early stress response of the kidney to acute injuries has revealed a number of potential biomarkers [4–7]. The bench-to-bedside journey of neutrophil gelatinase-associated lipocalin (NGAL), arguably the most promising novel AKI biomarker, is chronicled below.

Biology of NGAL

Human NGAL was originally identified as a 25-kDa protein covalently bound to gelatinase from neutrophils [8–10]. Like other lipocalins, NGAL forms a barrel-shaped tertiary structure with a hydrophobic calyx that binds small lipophilic molecules [11]. The major ligands for NGAL are siderophores, small iron-binding molecules. On the one hand, siderophores are synthesized by bacteria to acquire iron, and NGAL exerts a bacteriostatic effect by depleting siderophores. On the other hand, siderophores produced by eukaryotes participate in NGAL-mediated iron shuttling that is critical to various cellular responses such as proliferation and differentiation [11]. Although NGAL is expressed only at very low levels in several human tissues, it is markedly induced in injured epithelial cells, including the kidney [10]. The promoter region of the NGAL gene contains binding sites for a number of transcription factors, including NF-κB [8,9]. NF-κB is known to be rapidly activated in kidney tubule cells after acute injuries [12] and plays a central role in controlling cell survival and proliferation [13]. These findings provide a potential molecular mechanism for the documented role of NGAL in enhancing the epithelial phenotype, both during kidney development and following AKI [10].

NGAL for the early diagnosis of AKI

Preclinical transcriptome profiling studies identified *NGAL* (also known as lipocalin 2 or *lcn2*) to be one of the most upregulated genes in the kidney very early after acute injury in animal models [14–17]. Downstream proteomic analyses also revealed NGAL to be one of the most highly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models [18–20]. The serendipitous finding that NGAL protein was easily detected in the blood and urine soon after AKI has initiated a number of translational studies to evaluate NGAL as a non-invasive biomarker in human AKI. In a cross-sectional study, adults with established AKI (doubling of serum creatinine) displayed a marked increase in urine and serum NGAL by western blotting when compared to normal controls [20]. Urine and serum NGAL levels correlated with serum creatinine, and kidney biopsies in subjects with AKI showed intense accumulation of immunoreactive NGAL in cortical tubules, confirming NGAL as a sensitive index of established AKI in humans.

A number of studies have now implicated NGAL as an early diagnostic biomarker for AKI in common clinical situations. In prospective studies of children, with normal kidney function and no comorbid conditions, who underwent elective cardiac surgery, AKI (defined as a 50% increase in serum creatinine) occurred in ∼30% of the subjects, 2–3 days after surgery [21–23]. In contrast, NGAL measurements by ELISA revealed a 10-fold or more increase in the urine and plasma, within 2–6 h of the surgery in those who subsequently developed AKI. Both urine and plasma NGAL were excellent independent predictors of AKI, with an area under the curve (AUC) of >0.9 for the 2–6-h urine and plasma NGAL measurements [21–23]. These findings have now been confirmed in prospective studies of adults who developed AKI after cardiac surgery, in whom urinary NGAL was significantly elevated by 1–3 h after the operation [24,25]. AKI, defined as a 50% increase in serum creatinine, did not occur until 2–3 days later. The AUCs for the prediction of AKI were in the 0.71–0.80 range, the somewhat inferior performance perhaps reflective of confounding variables such as old age, pre-existing kidney disease, prolonged bypass times, chronic illness and diabetes [25].

NGAL has also been evaluated as a biomarker of AKI in kidney transplantation. Protocol biopsies of kidneys obtained 1 h after vascular anastomosis revealed a significant correlation between NGAL staining intensity and the subsequent development of delayed graft function [26]. In a prospective multicenter study of children and adults, urine NGAL levels in samples collected on the day of transplant identified those who subsequently developed delayed graft function (which typically occurred 2–4 days later), with an AUC of 0.9 [27]. Plasma NGAL measurements have also been correlated with delayed graft function following kidney transplantation from donors after cardiac death [28].

Several investigators have examined the role of NGAL as a predictive biomarker of nephrotoxicity following contrast administration [29–33]. In a prospective study of children undergoing elective cardiac catheterization with contrast administration, both urine and plasma NGAL predicted contrast-induced nephropathy (defined as a 50% increase in serum creatinine from baseline) within 2 h after contrast administration, with an AUC of 0.91–0.92 [33]. In several studies of adults administered contrast, an early rise in both urine (4 h) and plasma (2 h) NGAL was documented, in comparison with a much later increase in plasma cystatin C levels (8–24 h after contrast administration), providing further support for NGAL as an early biomarker of contrast nephropathy [30,31].

Urine and plasma NGAL measurements also represent early biomarkers of AKI in the pediatric intensive care setting, being able to predict this complication ∼2 days

prior to the rise in serum creatinine, with high sensitivity and AUCs of 0.68–0.78 [34,35]. In a recent study of adults in the emergency department setting, a single measurement of urine NGAL at the time of initial presentation predicted AKI with an outstanding AUC of 0.95, and reliably distinguished prerenal azotemia from intrinsic AKI and from chronic kidney disease (CKD) [36]. Thus, NGAL is a useful early AKI marker that predicts development of AKI even in heterogeneous groups of patients with multiple comorbidities and unknown timing of kidney injury.

Because of its high predictive properties for AKI, NGAL is also emerging as an early biomarker in interventional trials. For example, a reduction in urine NGAL has been employed as an outcome variable in clinical trials demonstrating the improved efficacy of a modern hydroxyethylstarch preparation over albumin or gelatin in maintaining renal function in elderly cardiac surgery patients [37,38]. Similarly, the response of urine NGAL was attenuated in adult cardiac surgery patients who experienced a lower incidence of AKI after sodium bicarbonate therapy when compared to sodium chloride [39]. Furthermore, adults who developed AKI after aprotinin use during cardiac surgery displayed a dramatic rise in urine NGAL in the immediate post-operative period, attesting to the potential use of NGAL for the prediction of nephrotoxic AKI [40]. Not surprisingly, NGAL measurements as an outcome variable are currently included in at least 10 ongoing clinical trials formally listed in ClinicalTrails.gov. The approach of using NGAL as a trigger to initiate and monitor novel therapies, and as a safety biomarker when using potentially nephrotoxic agents, is expected to increase.

The results described thus far have been obtained using research-based assays, which are not practical in the clinical setting. In this regard, a major advance has been the development of a standardized point-of-care kit for the clinical measurement of plasma NGAL (Triage® NGAL Device, Biosite Inc., San Diego, CA, USA). In children undergoing cardiac surgery, the 2-h plasma NGAL measurement measured by the Triage® Device showed an AUC of 0.96, sensitivity of 0.84 and specificity of 0.94 for the prediction of AKI using a cutoff value of 150 ng/ml [41]. The assay is facile with quantitative results available in 15 min, and requires only microliter quantities of whole blood or plasma. In addition, a urine NGAL immunoassay has been developed for a standardized clinical platform (ARCHITECT® analyzer, Abbott Diagnostics, Abbott Park, IL, USA). In children undergoing cardiac surgery, the 2-h urine NGAL measurement by ARCHITECT® analyzer showed an AUC of 0.95, sensitivity of 0.79 and specificity of 0.92 for prediction of AKI using a cutoff value of 150 mg/ml [42]. This assay is also easy to perform with no manual pretreatment steps, a first result available within 35 min, and requires only 150 μl of urine. Both kits are currently undergoing multicenter validation in adult populations.

NGAL for the prognosis of AKI

Recent studies have demonstrated the utility of early NGAL measurements for predicting clinical outcomes of AKI. In children undergoing cardiac surgery, the 2-h post-operative plasma NGAL levels measured by Triage® Device strongly correlated with duration and severity of AKI, and length of hospital stay. In addition, the 12-h plasma NGAL strongly correlated with mortality [41]. Similarly, the 2-h urine NGAL levels measured by ARCHITECT® analyzer highly correlated with duration and severity of AKI, length of hospital stay, dialysis requirement and death [42]. In a multicenter study of children with diarrhea-associated hemolytic uremic syndrome, urine NGAL obtained early during the hospitalization predicted the severity of AKI and dialysis requirement with high sensitivity [43]. Early urine NGAL levels were also predictive of duration of AKI (AUC 0.79) in a heterogeneous cohort of critically ill subjects [34]. In adults undergoing cardiopulmonary bypass, those who subsequently required renal replacement therapy were found to have the highest urine NGAL values upon arrival in the intensive care unit [25]. In adult kidney transplant patients undergoing either protocol biopsies or clinically indicated biopsies, urine NGAL measurements were found to be predictive of tubulitis or other tubular pathologies [44], raising the possibility of NGAL representing a non-invasive screening tool for the detection of tubulo-interstitial disease in the early months following kidney transplantation.

Sources of urinary and plasma NGAL

The genesis and sources of plasma and urinary NGAL following AKI require further clarification. Although plasma NGAL is freely filtered by the glomerulus, it is largely reabsorbed in the proximal tubules by efficient megalin-dependent endocytosis [11]. Direct evidence for this notion is derived from systemic injection of labelled NGAL, which becomes enriched in the proximal tubule but does not appear in the urine in animals [20]. Thus, any urinary excretion of NGAL is likely only when there is concomitant proximal renal tubular injury that precludes NGAL reabsorption and/or increases *de novo* NGAL synthesis. However, gene expression studies in AKI have demonstrated a rapid and

Table 1. NGAL as an AKI biomarker

Biomarker property	NGAL
Specific to AKI (AKI versus CKD versus systemic disease)	+/−[a]
Discern AKI sub-types (pre-renal azotemia versus intrinsic AKI)	Yes
Sensitive to establish an early diagnosis	Yes
Conserved across species	Yes
High gradient to allow early and easy detection	Yes
Proportional increase with injury or loss of function	Yes
Associated with a known mechanism	Yes
Results available while damage is limitable	Yes
Results predict clinical outcomes	Yes
Practical to measure	Yes
Amendable to existing platform assay methods	Yes

[a]Plasma NGAL may be detected in chronic kidney disease (CKD), chronic hypertension, systemic infections, inflammatory conditions and malignancies [51–56]. Urine NGAL may be detected in CKD, lupus nephritis and urinary tract infections [57–60]. In all these situations, NGAL values are generally substantially blunted compared to those typically measured in AKI.

Table 2. Urinary biomarkers for the early prediction of AKI in various clinical settings

Biomarker name and property	Cardiopulmonary bypass (CPB)	Contrast-induced nephropathy	Delayed graft function (DGF)	Intensive care or emergency setting
NGAL	2 h post-CPB 2 days pre-AKI	2 h post-contrast 1–2 days pre-AKI	12 h post-transplant 2–3 days pre-DGF	2 days pre-AKI
AUC	0.78–0.99	0.91–0.92	0.90	0.78–0.95
References	[21–25,41,42]	[29–33]	[27,28]	[34–36]
IL-18	12 h post-CPB 1–2 days pre-AKI	Not increased	12 h post-transplant 2–3 days pre-DGF	2 days pre-AKI
AUC	0.75		0.90	0.73
References	[22]		[27]	[64]
KIM-1	12 h post-CPB 1–2 days pre-AKI	Not tested	Not tested in acute setting	Not tested in acute setting
AUC	0.83			
References	[65]			

NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18; KIM-1, kidney injury molecule 1; AUC, area under the receiver operating characteristic curve.
AKI is defined as a 50% or greater increase in serum creatinine from baseline and DGF is defined as dialysis requirement within the first week after transplant. Times shown are the earliest time points when the biomarker becomes significantly increased from baseline.

Table 3. Urinary biomarkers for the early prediction of clinical outcomes in various AKI settings

Biomarker name	Cardiopulmonary bypass (CPB)	Kidney transplant	Intensive care or emergency setting
NGAL	Predicts AKI duration, severity, dialysis and death	Predicts AKI duration	Predicts AKI duration, severity and dialysis
References	[41,42]	[27]	[34,36,43]
IL-18	Predicts AKI duration	Predicts AKI duration	Predicts death
References	[22]	[27]	[66]
KIM-1	Not tested	Predicts long-term graft loss	Predicts dialysis and death
References		[67]	[68]

NGAL, neutrophil gelatinase-associated lipocalin; IL-18, interleukin 18; KIM-1, kidney injury molecule 1.
AKI is defined as a 50% or greater increase in serum creatinine from baseline.

massive (1000-fold) upregulation of NGAL mRNA in the thick ascending limb of Henle's loop and the collecting ducts [11]. The resultant synthesis of NGAL protein in the distal nephron and secretion into the urine appears to comprise the major fraction of urinary NGAL. Supporting clinical evidence is provided by the consistent finding of a high fractional excretion of NGAL reported in human AKI studies [11,20]. The over-expression of NGAL in the distal tubule and rapid secretion into the lower urinary tract is in accord with its teleological function as an antimicrobial strategy. It is also consistent with the proposed role for NGAL in promoting cell survival and proliferation, given the recent documentation of abundant apoptotic cell death in distal nephron segments in several animal and human models of AKI [45–48].

What about plasma NGAL in AKI? The kidney itself does not appear to be a major source, since direct ipsilateral renal vein sampling after unilateral ischemia indicates that the NGAL synthesized in the kidney is not introduced efficiently into the circulation, but is abundantly present in the ipsilateral ureter [11]. However, it is now well known that AKI results in a dramatically increased NGAL mRNA expression in distant organs [49], especially the liver and lungs, and the over-expressed NGAL protein released into the circulation may constitute a distinct systemic pool. Additional contributions to the systemic pool in AKI may derive from the fact that NGAL is an acute phase reactant and may be released from neutrophils, macrophages and other immune cells [50]. Furthermore, any decrease in glomerular filtration rate resulting from AKI would be expected to decrease the renal clearance of NGAL, with subsequent accumulation in the systemic circulation. The relative contribution of these mechanisms to the rise in plasma NGAL after AKI remains to be determined.

Limitations of NGAL as an AKI biomarker

Clearly, NGAL represents a novel predictive biomarker for AKI and its outcomes. However, the majority of studies published thus far have involved relatively small numbers of subjects from single centers, in which NGAL appears to be most sensitive and specific in homogeneous patient populations with predictable forms of AKI. Plasma NGAL measurements may be influenced by a number of coexisting variables such as CKD, chronic hypertension, systemic

infections, inflammatory conditions and malignancies [51–56]. In the CKD population, NGAL levels correlate with the severity of renal impairment [55,56]. However, the increase in plasma NGAL in these situations is generally much less than that typically encountered in AKI.

There is an emerging literature suggesting that urine NGAL is also a marker of CKD and its severity [1]. In subjects with CKD due to glomerulonephritides, urine NGAL levels were elevated and significantly correlated with serum creatinine, GFR and proteinuria [57]. In patients with autosomal dominant polycystic kidney disease, urine NGAL measurements correlated with residual GFR and severity of cystic disease [53]. Urine NGAL has also been shown to represent an early biomarker for the degree of chronic injury in patients with IgA nephropathy [58] and lupus nephritis [59,60], and may be increased in urinary tract infections. However, the levels of urine NGAL in these situations are significantly blunted compared to those typically measured in AKI.

Summary

NGAL as an AKI biomarker has successfully passed through the pre-clinical, assay development and initial clinical testing stages of the biomarker development process. It has now entered the prospective screening stage, facilitated by the development of commercial tools for the measurement of NGAL in large populations across different laboratories. But will any single biomarker such as NGAL suffice in AKI? In addition to early diagnosis and prediction, it would be desirable to identify biomarkers capable of discerning AKI subtypes, identifying etiologies, predicting clinical outcomes, allowing for risk stratification and monitoring the response to interventions. In order to obtain all of this desired information, a panel of validated biomarkers may be needed. The current status of NGAL as an AKI biomarker is shown in Table 1. Other AKI panel candidates may include interleukin-18 (IL-18), kidney injury molecule-1 (KIM-1), cystatin C and liver-type fatty acid binding protein (L-FABP), to name a few [4–6]. A brief comparison of the properties of NGAL versus other promising urinary biomarkers is shown in Tables 2 and 3.

The availability of a panel of AKI biomarkers could revolutionize renal and critical care. However, such idealistic thinking must be tempered with the enormous technical and fiscal issues surrounding the identification, validation, commercial development and acceptance of multi-marker panels. Deriving from the recent cardiology literature, a clinically useful biomarker should (a) be easily measurable at a reasonable cost with short turnaround times; (b) provide information that is not already available from clinical assessment; and (c) aid in medical decision making [61]. In this respect, troponin as a stand-alone biomarker provides excellent diagnostic and prognostic information in acute coronary syndromes and acute decompensated heart failure [62], although the addition of brain natriuretic peptide does improve the risk stratification of death from cardiovascular causes [63]. If the current prospective multicenter studies of NGAL measurements with standardized laboratory platforms provide promising results, we may have already closed in on the 'renal troponin'.

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A little help from our friends: what an epidemiologic study teaches us about autoinflammation, granuloma and proteinase-3-specific antineutrophil cytoplasmic autoantibodies

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The standard epidemiologic approach to complex diseases tracks down differences in incidence and prevalence rates between distinct populations. Thereby, the potential impact of genetic susceptibility and/or environmental factors will be elucidated and can be dissected on the molecular biologic level in further studies. In this journal issue of *Nephrology Dialysis Transplantation*, Watts *et al.* [1] report on the incidence of renal vasculitis in a population from the Norwich area, UK. The authors compared these data on renal involvement in the three anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and Churg Strauss syndrome (CSS), to recently published incidence rates of a Japanese population [1,2]. The overall incidence rate of renal vasculitis was similar in the UK and Japan ($12.2/10^6$ versus $14.8/10^6$). The incidence of WG ($5.8/10^6$) in the UK was slightly lower, that of MPA ($4.9/10^6$) slightly higher and that of CSS ($1.4/10^6$) comparable to that of newly diagnosed WG, MPA and CSS patients in central Europe [1,3]. However, no WG or CSS patients were seen between 2000 and 2004 in the Japanese study. All patients with renal vasculitis were diagnosed to suffer from MPA (incidence $14.8/10^6$). ANCA with a cytoplasmic fluorescence pattern (C-ANCA) and proteinase-3-specific (PR3)-ANCA were not detected among renal vasculitis patients in Japan. ENT involvement was virtually absent and neurological involvement was significantly less frequently diagnosed in renal vasculitis patients from Japan as compared to those from the UK [1,2].

Caveats with respect to this study regard the comparison of data from a prospective (UK) and a retrospective (Japan) study (as pointed out by the authors), comparing data from a hospital-based survey (single referral centre in Norwich, UK) with a population-based analysis of the incidence of AAV (Miyazaki Prefecture, Japan), and the lack of information, how ENT, respiratory, neurological and gastrointestinal involvement were determined. For instance, the history or a questionnaire on ENT-involvement could be biased by memory, attention and other reasons. Inspection with or without further endoscopic viewing by an ENT specialist plus a MRT scan of the head demonstrating signal intensity suggestive of inflammatory tissue in the sinuses and further signs of vasculitis discloses previously unsuspected and unrecognized involvement of

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