Albuminuria increases cystatin C excretion: implications for urinary biomarkers

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Abstract

Background. Low-molecular weight (LMW) proteins, including albumin and novel urinary biomarkers of acute kidney injury (AKI) such as cystatin C and neutrophil gelatinase-associated lipocalin (NGAL), are normally absorbed from the glomerular filtrate by receptor-mediated transport. We evaluated the effect of albuminuria on urinary excretion of novel biomarkers.

Methods. Sprague–Dawley rats given four injections over 2 days of 5 mg/g body wt/day bovine serum albumin (BSA) in saline were compared with controls given saline alone. Urinary cystatin C, albumin and protein excretion rates were compared prior to treatment (Day −1), after treatment (Day 2) and 4 days later (Day 6). A preliminary assessment of the clinical effect of proteinuria on the filtered urinary biomarkers cystatin C and NGAL was made by comparison with the effect on urinary interleukin-18 (IL-18) that is not absorbed from the glomerular filtrate, in a cohort of intensive care unit patients.

Results. BSA induced transient increases in albuminuria, proteinuria and cystatinuria (P < 0.01, P < 0.001 and P < 0.001, respectively). Beyond a threshold 6-fold increase in albuminuria, cystatin C absorption was reduced by competitive inhibition. The excretion rates of all analytes returned to preinjection levels by Day 6. Clinical proteinuria was associated with increasing cystatin C and NGAL concentrations (n = 90, P < 0.0001) but not IL-18 (P = 0.12).

Conclusions. Proteinuria may increase the threshold for detection of AKI by increasing the excretion of LMW protein biomarkers.

Keywords: acute kidney injury; albumin; biomarkers; cystatin C; proteinuria

Introduction

Recent studies have highlighted the potential for early detection of acute kidney injury (AKI), and for triaging to early intervention by urinary biomarkers [1–3]. Several of these biomarkers are low-molecular weight (LMW) proteins that appear in urine following ischaemic or toxic renal injury [3]. Urinary cystatin C (CysC) and neutrophil gelatinase-associated lipocalin (NGAL) are important examples of such biomarkers. CysC and NGAL increase prior to plasma creatinine (pCr) in cardiac surgery-associated AKI and in general intensive care patients [4–8].

CysC is a 13.3 kDa, 122 amino acid polypeptide, produced at a constant rate by all nucleated cells [9]. It has a protective role both intracellularly, as a cysteine protease inhibitor, and extracellularly, as a lysosomal proteinase inhibitor [10]. CysC is distributed only in the extracellular volume and cleared by glomerular filtration without tubular secretion [11] with a half-life of 1.5 h [12]. Consequently, the increase in plasma CysC concentrations after acute changes in the glomerular filtration rate (GFR) are faster and reach steady state sooner than creatinine [13, 14].

Filtered CysC is reabsorbed by megalin-facilitated endocytosis in proximal tubules and catabolized [15]. Consequently, proximal tubular injury in AKI will reduce reabsorption and produce a diagnostic increase in urinary CysC [15]. However, filtered albumin is also reabsorbed by megalin–cubulin receptor-mediated endocytosis [16, 17]. Increased urinary CysC has been observed in the presence of proteinuria in children with nephrotic syndrome [18]. Independently of tubular injury, competition for receptor-mediated transport between albumin and other LMW proteins could account for or make a significant contribution to increased urinary CysC in the presence of proteinuria [19].

Other small size proteins or drugs absorbed by receptor-mediated endocytosis include other novel biomarkers of AKI, such as NGAL, liver fatty acid-binding protein (L-FABP), α1-microglobulin and β2-microglobulin [20–23]. We hypothesized that albuminuria could contribute to an increase in the concentration of AKI biomarkers independent of tubular injury. We investigated this by measuring urinary CysC excretion in a rat model of transient

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albuminuria induced by protein overload. We also examined whether this hypothesis had clinical support by performing a retrospective analysis of urinary biomarkers in a cohort of critically ill patients in whom urinary protein concentrations were semiquantitatively measured by urinalysis.

Materials and method

Animal experiment

All animal experiments were approved by the Animal Ethics Committee, University of Otago, Christchurch, New Zealand. Rats were housed in cages (3–4 animals per cage) at 22 ± 2°C and exposed to a 12:12-h dark-light cycle. Rats were fed with standard rat chow (Weston Milling, Rangiora, New Zealand) and had unlimited access to food and water.

Sprague–Dawley–Hannover (SD) rats were used and induted into the study at 6–9 weeks of age. Rats were randomly assigned to treatment (n = 14) and control (n = 8) groups such that there were equal numbers of male and female rats in each group. For two days, treated rats received twice daily intraperitoneal (IP) injection of 5 mg/g body wt/day bovine serum albumin (BSA) (BSA fraction v, No. A-4503, 96–99% albumin, Sigma Chemical Company, St. Louis, MO) dissolved in 2.5 mL of 0.9% NaCl to induce overload proteinuria [24, 25]. Control animals received IP injections of equal volumes of 0.9% saline on an identical schedule. SD rats were chosen because induction of proteinuria following IP injection of BSA has been observed previously in these animals [24, 25]. The protocol followed that described by Pippin [24], except that IP injections were discontinued after the fourth injection at 48 h.

Metabolic caging (Tecniplast, Rydalmere NSW, Australia) was undertaken on the day before albumin treatment commenced (Day −1), the day following the final supplementation (Day 2) and 96 h later (Day 6). Each urine collection was from 5 pm until 9 am the following day. Rats were weighed before and after metabolic caging. Albumin, total protein, creatinine and CysC concentrations were measured in the timed collections (16 h) of spontaneously voided urine.

Tail vein sampling was used to collect blood (0.75 mL) into heparinized tubes prior to each urine collection (by overnight metabolic caging). Plasma was separated immediately and stored at −20°C for subsequent creatinine and CysC analysis.

Pcr was measured by high-performance liquid chromatography using a protocol adapted from Van Landuyt et al. [26]. Urinary creatinine was measured by the Jaffe method using the Beckmann Creatinine Analyzer. Creatinine clearance was calculated using the formula: (urinary creatinine concentration/Pcr) × (total urine volume/time). Urinary albumin concentration was measured by competitive enzyme-linked immunosorbent assay based on the method of Chen et al. [27]. The concentration of CysC in urine and plasma was quantified using a BNII nephelometer (Dade Behring Inc)[28, 29]. The rates of protein, albumin and CysC excretion in urine were calculated for each metabolic caging by multiplying the urinary concentration by the total urine volume divided by time.

The CysC absorption rate was estimated from the difference between the rates of CysC excretion and filtration (estimated as plasma CysC×creatinine clearance).

The affinity for CysC absorption (Michaelis–Menten coefficient, Km) was derived from a Lineweaver–Burke plot of the reciprocal of CysC absorption rate against the reciprocal of the estimated CysC filtration concentration (estimated as the same as the plasma concentration, pCysC). The filtration concentration data were transformed by subtraction of a constant so that x = 0 coincided with the intercept of the linear regression lines for all animals on Day −1 and for BSA-treated animals on Day 2. This transform is based on the assumption that the maximum velocity of the reaction was the same for Day −1 and Day 2 (since intersection of the linear regressions of the two groups was observed in the Lineweaver–Burke plot). The Michaelis–Menten coefficients on Day −1 and Day 2 were determined from the intercept of the regression lines at y = 0.

Clinical study

Proteinuria data was retrospectively analyzed in patients in the EARLYFRC combined observational study of urinary biomarkers and early intervention in AKI in the intensive care unit (ICU) [2, 30]. Since the decision to evaluate proteinuria was taken after the study had commenced, semiquantitative analysis was performed by dipstick analysis only on samples in one of the two centers participating in the study.

Dipstick analysis was carried out in 91 of 322 patients. This analysis was performed prospectively on the last 29 patients enrolled in the study. The remaining data (n = 62) was collected retrospectively after identification of those patients who had dipstick urinalysis following admission to ICU.

Urine samples were collected within 1 h of ICU admission for measurement of urinary biomarkers including, CysC and NGAL. Proteinuria was detected by test strip (Bayer Reagent) and reported according to CysC excretion (0 [none], 1+, 2+, 3+, 4+). CysC, NGAL and interleukin-18 (IL-18) were assayed as previously described [30]. Briefly, CysC was measured using the immunonephelometric assay already described; NGAL was measured using the NGAL ELISA Kit 036 (Antibody Shop, Grubskabken, Denmark) [31]. IL-18 was measured using a human IL-18 ELISA kit (Medical and Biological Laboratories, Nagoya, Japan) that detects the mature form of IL-18 [32]. AKI was defined as an increase of >0.3 mg/dL or 50% above an adjudicated baseline as previously described [30, 33].

Statistical analyses

Two-way repeated measures analysis of variance (ANOVA) was performed on the results from each urine analyte. Main effects and/or interactions were further explored using Fisher’s Protected Least Significant Difference (LSD test). Pearson’s correlations between absolute changes in albumin, protein and CysC excretion (Day 2 minus Day −1 excretion rates) were calculated. Data were transformed where necessary using the natural logarithm. Data are presented as mean ± SEM or median (inter-quartile range) as appropriate. For the analysis of the association of proteinuria with CysC and NGAL in ICU patients, CysC and NGAL concentrations were log transformed and assessed by one-way ANOVA. Statistical analyses were performed in SPSS (version 16, SPSS Inc., Chicago, IL) and GraphPad Prism (version 5.0a; GraphPad Software, San Diego, CA).

Results

Rats gained weight normally during the experiment and to a similar extent to colony rats, suggesting minimal stress.

The rate of protein excretion (mg/hour) increased in the BSA-treated group from 0.48 ± 0.12 (mean ± SEM) to 1.4 ± 0.32 (Day −1 compared with Day 2, P < 0.01; Figure 1A). At the same time, the rate of albumin excretion (mg/hour) increased 10-fold from 0.04 ± 0.02 to 0.43 ± 0.15 (P < 0.05; Figure 1B), and the rate of CysC excretion (μg/hour) increased from 0.02 ± 0.004 to 0.05 ± 0.008 (P < 0.001; Figure 1C). The protein excretion rate was correlated with the albumin excretion rate (r² = 0.72, P < 0.0001; Figure 2A). The change in the rate of CysC excretion from Day −1 to Day 2 correlated with the change in rates of albumin excretion (r² = 0.29, P < 0.01; Figure 2C) and protein excretion (r² = 0.58, P < 0.0001; Figure 2B). As there were no differences between the excretion rates of protein, albumin and CysC at Day −1 and Day 6 (P = 0.61, P = 0.61 and P = 0.21, respectively), the functional changes induced by BSA overload were transient. The rates of excretion of protein, albumin and CysC depended on gender. Male rats excreted more protein, albumin and CysC than female rats both prior to and after injections (P < 0.0001, P < 0.0001 and P < 0.01, respectively).

Creatinine clearance was unchanged by BSA treatment (Day −1 compared with Day 2). For the BSA-treated group, creatinine clearance (mean ± SEM; mL/hour) did not change from 103 ± 7 (Day −1) to 107 ± 7 (Day 2) (P = 0.31). There was no change in plasma CysC concentrations from Day −1 to Day 2 in BSA-treated animals.
Saline-treated rats showed no changes in urine analytes over time.

The apparent Michaelis–Menten coefficient (Km) for CysC absorption increased from $12 \pm 9 \text{ nM} \ (0.16 \pm 0.12 \text{ mg/L})$ on Day $-1$ to $21 \pm 12 \text{ nM} \ (0.27 \pm 0.16 \text{ mg/L})$ on Day 2 ($P < 0.05$) following BSA treatment (Figure 3) consistent with competition by albumin. The nonparallel shift in absorption rate supports competitive inhibition. In BSA-treated rats, the ratio of albumin concentration on Day 2/Day $-1$ versus the ratio of urinary CysC excretion and absorption at these time points showed that there was an >6-fold increase in albumin concentration before a change in CysC absorption or excretion (Figure 4). The CysC absorption rate halved after an ~16-fold increase in albumin concentration.

Fig. 1. Rate of excretion of protein (A), albumin (B) and Cystatin C (C) on Day $-1$ (preinjection), Day 2 (the day following the last IP injection) and Day 6 (96 h after Day 2). Histograms show the mean and error bars the SE. *Indicates the significant differences between Day 2 and Day $-1$ (*, $P < 0.05$; **, $P < 0.01$).

Fig. 2. Change in albumin and Cystatin C excretion rates from Day $-1$ to Day 2 as a function of the difference in protein and albumin excretion rates. (A) albumin versus protein excretion rate difference; (B) Cystatin C versus protein excretion rate difference; (C) Cystatin C versus albumin excretion rate difference.
Dipstick urinalysis was available for 91 patients from the EARLY ARF trial (Table 1). These patients did not differ statistically in age, weight, sex, APACHE II score, SOFA score, baseline pCr or estimated GFR or presence of sepsis from those without dipstick analysis. Biomarker concentrations were higher in AKI than non-AKI patients: CysC [AKI: 1.0 (0.09–4.8) mg/dL, non-AKI: 0.16 (0.05–1.71) mg/dL, \( P = 0.034 \)], NGAL [AKI: 338 (39–2250) ng/mL, non-AKI: 42 (13–225) ng/mL, \( P = 0.0027 \)], IL-18 [AKI: 361 (9.2–988) pg/mL, non-AKI: 0.001 (0.001–321) pg/mL, \( P = 0.0011 \)]. The proportion of patients with AKI trended higher with higher grade of proteinuria: 19% (0), 18% (1+), 40% (3+), 50% (4+), \( P = 0.019 \). The concentrations of CysC and NGAL, but not IL-18, were associated with degree of proteinuria: CysC (\( n = 90 \), \( P < 0.0001 \)); NGAL (\( n = 85 \), \( P < 0.0001 \)); (\( n = 91 \), \( P = 0.12 \)) (Figure 5). A post hoc analysis using pairwise comparison of log-transformed means showed significant differences between no protein (0) and 2+, 3+ and 4+, between 1+ and 2+, 3+ and 4+ for CysC, and between 0 and 2+, 3+ and 4+ and between 1+ and 3+ for NGAL.

Discussion

This study demonstrated that repeated protein loading induces transient proteinuria and albuminuria, as shown previously [24], and that rats with induced albuminuria and proteinuria produced parallel increases in rates of urinary CysC excretion. Importantly, urinary CysC excretion decreased again when albuminuria and proteinuria returned to baseline. These observations are consistent with the hypothesis that albuminuria decreases the absorption of LMW proteins by competition for common transport mechanisms [19]. The association between proteinuria and urinary CysC and NGAL in the available sample of ICU patients in the EARLY ARF trial supports the hypothesis that LMW proteins compete for reabsorption and suggests that the presence of proteinuria may affect the threshold for observation of urinary LMW protein biomarkers after AKI.

Mechanisms contributing to proteinuria following protein injection include increased podocyte pinocytosis and lysosomal activity, which lead to formation of vacuoles and absorption of droplets [34, 35]. Following swelling of podocytes, foot processes partially lift from the glomerular basal membrane and then completely detach [36]. Most of these changes are reversible. If no permanent damage has occurred.

Table 1. Demographics of the ICU patient cohort (\( n = 91 \))

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
<th>Median (Q1-Q3)</th>
<th>( n )</th>
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<tr>
<td>Age, years</td>
<td>60 ± 17</td>
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<tr>
<td>Female</td>
<td>34 (37%)</td>
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<td>APACHE II</td>
<td>19 ± 7</td>
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<td>SOFA</td>
<td>6.5 ± 2.9</td>
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<td>Baseline plasma creatinine, mg/dL</td>
<td>0.91 (0.68–1.0)</td>
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<td>Baseline eGFR, mL/min</td>
<td>87 (66–106)</td>
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<tr>
<td>CKD (eGFR &lt;60 mL/min)</td>
<td>18 (20%)</td>
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<tr>
<td>Sepsis</td>
<td>18 (20%)</td>
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<td>AKI on entry(^a)</td>
<td>34 (37%)</td>
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<tr>
<td>Death in 7 days</td>
<td>9 (10%)</td>
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<tr>
<td>NGAL on entry, ng/mL</td>
<td>79 (13–437)</td>
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<tr>
<td>Cystatin C on entry, mg/L</td>
<td>0.23 (0.07–2.1)</td>
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<td>IL-18 on entry, pg/mL</td>
<td>62 (0.001–605)</td>
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<td>Proteinuria</td>
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<td>4+</td>
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\(^a\)Presented are mean ± SD for normally distributed continuous variables or median (lower quartile-upper quartile) for not normally distributed continuous variables, \( n \) (%) for categorical variables.

\(^b\)AKI: >0.3 mg/dL or 50% increase in plasma creatinine above baseline.

eGFR, estimated GFR; CKD, chronic kidney disease.
occurred urinary excretion of protein regresses and normalizes once protein overload ceases [24]. In this study, proteinuria and albuminuria returned to control values within 4 days. This suggests that no permanent change was induced either to the glomerular protein filtration or to the tubular absorption mechanism [37]. Additionally, plasma creatinine concentration and creatinine clearance were unaltered during the induction of proteinuria.

Competition for absorption between albumin and LMW proteins was first reported by Bernard et al. [38, 39]. An intravenous injection of BSA was reported to inhibit absorption of β2-microgloblin [38, 39] and attributed to the higher receptor affinity of albumin compared with β2-microgloblin [40]. Thielemans et al. [19] induced albuminuria by IP injection of puromycin aminonucleoside (PAN) and adriamycin, which are known to affect the glomerulus, or mercuric chloride and maleic acid, which are known to injure proximal tubules. Thielemans et al. demonstrated that injury by either mechanism was accompanied by an increase in LMW proteins, presumably as a result of competition for a common transport mechanism. More recently, it has been confirmed that albumin is transported by receptor-mediated endocytosis, which is cooperatively facilitated by the highly expressed apical receptor proteins megalin and cubulin in proximal tubular cells [15–17, 21]. Megalin and cubulin receptors cooperatively facilitate absorption of virtually all LMW proteins, including albumin [17].

Dieterle et al. [20] recently demonstrated that increased urinary CysC and β2-microgloblin were diagnostic of glomerular injury in rats with drug-induced glomerular injury. However, part of the increase in these urinary biomarkers may have resulted from competition between these LMW proteins and albumin, since the glomerular toxin used was puromycin, which increases glomerular permeability to albumin [19]. Similar observations are potentially relevant to other urinary biomarkers that are filtered LMW proteins including NGAL, α1-microgloblin and L-FABP [22, 23].

Filtered NGAL is normally absorbed by proximal tubular cells through megalin transporters. In addition to detecting AKI, urinary NGAL has been reported as a marker of risk for chronic kidney disease (CKD) progression [41] and a predictor of worsening renal function in patients with proteinuria [42]. While albuminuria is known to result from increased permeability of the glomerular barrier, proximal tubular dysfunction may also cause albuminuria in acquired or chemically induced kidney diseases and in patients with diabetic nephropathy [43]. Albuminuria develops in the early stage of diabetic nephropathy and CKD [44]. Elevation of urinary NGAL has been reported in PAN-treated rats [45], suggesting that increased urinary NGAL may result from receptor saturation [46]. These conclusions are supported by the recent observation that serum and urine NGAL concentrations in diabetic children correlate with the albumin excretion rate [47]. Similarly, L-FABP, which is also absorbed by the megalin-cubulin pathway [23], has been shown to increase in the urine of diabetic patients [48], which may reflect either tubular injury or receptor saturation [48]. Ischemic or toxic injury to the proximal tubules could directly injure or modify the LMW protein transporters. Urinary α1-microgloblin and albumin are both elevated very early following cardiac surgery in children with AKI [49]. Microalbuminuria may occur acutely in critical illness, even in the absence of AKI, probably from changes in capillary permeability [50] or an
inflammatory response [51]. Hence, competition between albumin and urinary biomarkers for absorption by the same receptors may contribute to elevation of AKI biomarkers in the urine following cardiopulmonary bypass.

Our observation in a cohort of critically ill patients that the LMW urinary proteins CysC and NGAL were elevated in the presence of proteinuria supports this hypothesis, although we note that the post hoc analysis did not reveal a stepwise increase in concentrations with increasing grade of proteinuria. While IL-18, which does not appear to bind to megalin or cubulin, was not shown to be statistically significantly elevated by increasing proteinuria, at P = 0.12, we would caution that this is not strong evidence in support of the hypothesis. Some elevation of CysC, NGAL and urinary albumin may be expected in the presence of AKI because of concomitant damage to the proximal tubular cells and hence the LMW protein reabsorption mechanism. It follows that albuminuria in CKD patients may explain the poorer performance of NGAL for the prediction of AKI in this cohort [30, 52] and suggests that the performance of albumin itself should be assessed prospectively as a biomarker of AKI. One recent experimental study [53] of nephrotoxin-induced injury and the clinical study of children following cardiac surgery [49] found albumin to be an early biomarker of AKI. However, confounding the explanation for our clinical findings is that the urinary biomarker concentrations were performed in critically ill subjects at high risk for developing AKI (indeed, 37% already had AKI), at an early time point (on entry to the ICU). Urinary NGAL and CysC are known to be early responders to AKI and the early indications are that albumin is also an early responder, whereas urinary IL-18 is an intermediate responder in temporal sequence [49, 54, 55]. Furthermore, it is well known that the NGAL transcript and protein are massively upregulated in the distal tubule very early during the course of AKI [56]. Therefore, the increased urinary NGAL associated with increased urinary dipstick proteinuria reported herein may represent merely coincidental associations. Nevertheless, if we assume proximal tubule injury is focal in humans, competition in the remaining nephrons may still complicate the interpretation of the increase in biomarker.

By assuming that the proximal tubular concentration of CysC was equivalent to the plasma concentration, a reasonable but clinically unverifiable assumption, we approximated the affinity for CysC binding by megalin (K_m = 12 nM). Given the approximations required to estimate this coefficient, we caution that it should not be considered an exact measurement. However, after induction of proteinuria, the pattern of decrease in CysC absorption rate supports competitive inhibition of CysC transport. These observations suggest that interpreting an increase in urinary LMW protein biomarker concentrations requires an understanding of the degree to which the biomarker concentration is increased by competition for absorption by albumin.

Just as transporter affinity for CysC decreased in the presence of proteinuria, we presume that affinity will decrease for other LMW proteins transported by the megalin–cubulin pathway with similar receptor affinity. In a practical sense, this may simply require an increase in the threshold for diagnosis of AKI when proteinuria is present. This suggests that urinary biomarker performance should take the degree of albuminuria/proteinuria into account. NGAL binds to megalin receptors with a lower affinity (K_m = ~60 nM) [22]. This suggests that urinary NGAL excretion will be increased by proteinuria to a greater extent than CysC. While the fold range of increase in NGAL observed in our clinical cohort appears greater than for CysC (Figure 5), the semiquantitative measurement of proteinuria cannot confirm this. We note a further limitation in that this study was restricted to one of two centers, a small group of patients and because dipstick urinalysis was undertaken in a minority of patients, these may well have been subject to selection bias. Furthermore, we note the well-known limitations of measuring urine protein by dipstick (e.g. influence of urine pH, concentration and hematuria), and formal measurements of urinary albumin excretion will be required for confirmation of our hypothesis in human AKI.

The experimental data in rats suggest that proteinuria-induced increases in urinary LMW biomarker excretion may be common and independent of proximal tubular injury. The clinical data is less clear as a number of patients were likely to have had proximal tubular damage and it is not possible to tell how much of the increase in the biomarker is as a result of damage to proximal tubules and how much due to competition in the remaining nephrons. Nevertheless, this concern is compounded by the observation that AKI occurs on a background of CKD in nearly half the ICU population [57] and that proteinuria is common in CKD. Also, patients with glomerular disease have significant albuminuria and, therefore, are likely to have elevated LMW proteins. Intravenous administration of albumin may also induce albuminuria. It is likely that urinary biomarker performance in the diagnosis of AKI will be modified by the presence of filtered albumin. If the threshold for diagnosis is not modified by the presence of preexisting albuminuria, then there is the potential for an increase in the number of false positives. Urinary albumin itself is reported to be a biomarker of AKI [53, 58], presumably as a result of proximal tubular injury.

Three conclusions are suggested by these observations. Firstly, LMW urinary biomarker concentrations are affected by both competition for absorption and tubular injury. Secondly, since albuminuria may increase the threshold for detection of AKI using novel biomarkers, formal quantitation of albuminuria may facilitate interpretation of urinary biomarker performance. Thirdly, the recent observation that urinary CysC was predictive of sepsis in ICU patients [6], suggests that stratification for sepsis and other potential causes of ‘glomerular leak’ albuminuria, such as cardiovascular disease [59] may also facilitate AKI biomarker assessment.

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Conflict of interest statement. P.D. is a coinventor on patent applications covering the use of NGAL as a biomarker of chronic and acute kidney...
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