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Original Paper

Dexamethasone Modifies Cystatin C-Based Diagnosis of Acute Kidney Injury During Cisplatin-Based Chemotherapy

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Key Words
Cystatin C • Creatinine • Acute kidney injury • Biomarkers • Dexamethasone

Abstract
Background/Aims: Plasma cystatin C (pCysC) may be superior to serum creatinine (sCr) as a surrogate of GFR. However, the performance of pCysC for diagnosing acute kidney injury (AKI) after cisplatin-based chemotherapy is potentially affected by accompanying corticosteroid anti-emetic therapy and hydration. ***Methods:*** In a prospective observational study pCysC, sCr, urinary kidney injury molecule-1 (KIM-1), and urinary clusterin were measured over 2 weeks in 27 patients given first-cycle chemotherapy. The same variables were measured over 2 weeks in Sprague–Dawley rats given a single intraperitoneal injection of dexamethasone, cisplatin, or both, and in controls. ***Results:*** In patients, pCysC increases were greater than sCr [41% vs. 16%, mean paired difference 25% (95% CI: 16–34%)], relative increases were ≥ 50% in 9 patients (35%) for pCysC compared with 2 (8%) for sCr ($p = 0.04$) and increases in sCr were accompanied by increased KIM-1 and clusterin excretion, but increases in pCysC alone were not. In rats, dexamethasone administration produced dose-dependent increases in pCysC (and augmented cisplatin-induced increases in pCysC), but did not augment histological injury, increases in sCr, or KIM-1 and clusterin excretion. ***Conclusions:*** In the presence of dexamethasone, elevation of pCysC does not reliably diagnose AKI after cisplatin-based chemotherapy.

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Introduction

Acute kidney injury (AKI) is defined clinically by the presence of oliguria, or an increase in serum creatinine (sCr) of either ≥ 26.5 μmol/L (0.3mg/dL) within 48 hours, or by ≥ 50% of baseline values within 7 days of exposure to a renal insult [1, 2]. The limitations of sCr and the need for alternative AKI biomarkers have been documented repeatedly [1, 2] and precipitated the recommendation to measure both functional change and kidney damage in diagnosing AKI [2]. Estimated GFR (eGFR) using serum or plasma cystatin C (pCysC) improves the classification of chronic kidney disease [3], and a ≥ 50% increase in pCysC has been proposed to replace or complement a ≥ 50% increase in sCr in defining functional AKI [4-7].

pCysC has regulatory approval for use in pre-clinical models as a marker of toxicant-induced kidney injury including that caused by the cytotoxic agent cisplatin [4]. Cisplatin is commonly prescribed to treat numerous malignancies, despite an incidence of AKI of 4–23% [4]. The use of pCysC for diagnosis of AKI in patients treated with cisplatin and its relationship to structural markers of AKI has not been reported [4]. A particular concern in this setting is the corticosteroid based anti-emetic therapy and hydration that routinely accompanies cisplatin. Hydration affects the kinetics of sCr and pCysC [8-10] and corticosteroids can increase production of CysC [11-18].

Clusterin appears to be anti-apoptotic in kidney injury, and involved in cell protection, lipid recycling, cell aggregation and cell attachment. It is upregulated in renal tissues in both humans and experimental models by various forms of cellular stress including cisplatin-induced toxicity [4]. Upregulation of kidney injury molecule-1 (KIM-1) in proximal epithelial cells enables proximal tubule cells to recognize and phagocytose apoptotic cells [19]. The ectodomain of KIM-1 is shed from proximal tubular kidney epithelial cells into urine in rodents and humans after injury including cisplatin [4, 20]. Both clusterin [21] and KIM-1 [22] have been validated by the the US Food and Drug Administration (FDA) and European Medicines Agency (EMEA) for detection of acute drug-induced renal toxicity in rodent studies.

We conducted a prospective observational study in patients receiving cisplatin chemotherapy to evaluate the use of pCysC and the urinary kidney damage biomarkers KIM-1 and clusterin in the diagnosis of AKI. This was followed by an animal study which examined the effect of dexamethasone on these parameters and on a model of moderately-severe cisplatin-induced AKI in rats.

Materials and Methods

Clinical Study

The clinical study was approved (11/008) by the Human Research Ethics Committee at South Eastern Sydney Local Health District, New South Wales and conducted in adherence with the Declaration of Helsinki. Written informed consent was sought obtained from patients, 18 years or older, who were admitted to Prince of Wales Hospital, Sydney, for the first cycle of cisplatin-based chemotherapy for treatment of solid tumors. Exclusion criteria included inability to provide informed consent; eGFR <45 mL/min/1.73m^2; albuminuria > 300 mg/day; concurrent potentially nephrotoxic treatments including carboplatin, ifosfamide, non-steroidal anti-inflammatory drugs; concurrent or recent infection, and factors limiting outpatient sample collection.

All treatment was prescribed independently by treating physicians. Each patient was treated as an inpatient and received 2.0–2.6 L intravenous crystalloid over 3.25–3.75 h before cisplatin, and 4 L crystalloid over 13 h after cisplatin. Each patient also received a single oral dose of dexamethasone 8 mg, and aprepitant 165 mg, plus 250 μg intravenous palonosetron as standard anti-emetic prophylaxis.

Blood and urine were collected before intravenous hydration (Baseline); immediately before cisplatin (0h); and then at 4h, 8h, 1d, 3d, 7d and 14d after cisplatin commenced. Urine samples were promptly mixed 2:1 with protease-inhibitor cocktail (cOmplete, Roche Diagnostics, Mannheim, Germany) before

centrifugation (1000g, 8min) to remove debris. Aliquots of plasma, serum and urine were stored at -80°C and thawed at 37°C for batched assay. Repeated freeze-thaw cycles were avoided.

Creatinine and urate concentrations were measured using enzymatic methods on an automated chemical analyser (Konelab 20XT, Thermo Fisher Scientific, Waltham, MA, USA) and pCysC was measured using microparticle-enhanced immunoturbidometric methods using the same analyser and calibrated using standards (X791201, Dako Glostrup, Denmark) traceable to the International Federation of Clinical Chemistry Working Group certified reference material (ERM-DA471/IFCC) (4, 5).

Clusterin, and KIM-1 were measured in duplicate using DuoSet ELISA assays (R&D Systems, Minneapolis, MN, USA). The intra-assay and inter-assay variability for all assays was less than 10%.

The baseline eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations for sCr [1] and CysC (7, 8). Oliguria was defined by urine output <0.5 mL/h for ≥ 6h [1].

Animal Study

Male Sprague Dawley (SD) rats (205- 220g) were purchased from Animal Resources Centre, (Perth Western Australia) and maintained in a central animal facility under conditions of 21-23°C and 55–70% humidity in an alternating 12 h light-dark cycle. Rats were provided with commercial rodent chow and water *ad libitum*, and acclimatised for 1 week before experimentation. All animal maintenance and treatment protocols complied with the Australian Code for the Care and Use of Animals for Scientific Purposes and were approved by the University of New South Wales Animal Care and Ethics Committee (14/15A).

Rats were randomly assigned to dexamethasone (3 dose levels, n = 5 each), control (n = 5), cisplatin plus dexamethasone (n = 5), or cisplatin plus saline (n = 5). Simple allometric scaling [1] was used to estimate a dexamethasone dose for rats (0.5mg/kg) equivalent to the mean dose received in human subjects (0.09 mg/kg). On day 0 treated rats received dexamethasone 0.5 mg/kg, 2.5 mg/kg or 10 mg/kg diluted in 0.9% NaCl via a single intraperitoneal injection of 1.25 mL. Control rats received 1.25 mL intraperitoneal 0.9% NaCl. Cisplatin plus dexamethasone treated rats received cisplatin 6mg/kg (1mg/mL) 30 min prior to dexamethasone 2.5 mg/kg as above. Cisplatin plus saline treated rats received cisplatin 6mg/kg 30 min prior to 1.25 mL intraperitoneal 0.9% NaCl.

Blood and urine were collected before treatment (baseline) and then at 1d, 2d, 3d, 5d, 7d, 10d and 14d after treatment. On preceding nights, rats were placed in individual metabolic cages (Tecniplast, Buguggiate, VA, Italy) from 1600h to 0800h. Urine was collected on cooled blocks before prompt centrifugation (1000g, 8min) to remove debris. For blood collection and concurrent intraperitoneal injection each rat underwent brief isoflurane sedation and approximately 120 µL of blood was collected via tail vein venepuncture. At scheduled necropsy, rats received pentobarbitone sodium (50 mg/kg intraperitoneal single dose) and were exsanguinated via cardiac puncture. Collected blood was allowed to clot for 30 min and then centrifuged (1500 g, 8 min). Aliquots of serum and urine supernatant were promptly stored at -80 °C for batched analysis.

At necropsy, at 14d, a 5 mm coronal section including the left renal hilum was fixed in 10% neutral buffered formalin for 24 h, paraffin embedded, cut into 4 µm sections and stained with hematoxylin and eosin. Digital images were obtained using the ScanScope Digital Slide Scanner (Aperio, Vista, CA) at 40× magnification. Five fields of cortex, outer stripe of outer medulla, and inner stripe of outer medulla were arbitrarily captured. Fields were examined using ImageJ, (US National Institutes of Health, Bethesda, MD) blinded from biomarker data and the intervention group and reviewed by a second investigator.

Rat urine creatinine was measured as described above. To minimise the volume required, the serum assay was adapted to a 96-well microplate reader using otherwise identical methods. Rat CysC was measured with Quantikine ELISA (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

Rat KIM-1 and clusterin were measured using Bio-Plex Pro RBM Rat Kidney Toxicity Panel magnetic immunobeads (Bio-Rad Laboratories Hercules, CA, USA) on the Bio-Plex 200 system (Bio Rad Laboratories, Berkerly, CA, USA).

Statistical Methods

Statistical analysis was performed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA). All analyses were two-tailed.

The primary outcome was the difference in peak relative increase of sCr and pCysC in patients. The

relative increase of either circulating biomarker at any time was defined as the percentage change of the biomarker from baseline values. Primary analyses for urinary biomarkers were conducted on concentrations normalised to urinary creatinine to allow for differences in hydration status and urine output (*i.e.* "creatinine-normalised"). As suggested elsewhere secondary analyses used absolute concentrations of biomarkers [23].

Secondary analyses included categorising AKI according to whether patients had a ≥ 50% increase in sCr **and** pCysC (denoted AKI-Cr/CysC), a ≥ 50% increase in sCr without a similar increase in pCysC (AKI-sCr), a ≥ 50% increase in pCysC without a similar increase in sCr (AKI-pCysC), or a ≥ 50% increase in neither sCr nor pCysC (No-AKI). Patients experiencing "AKI-Cr/CysC" or "AKI-sCr" fulfiled the KDIGO 2012 criteria for AKI [1] based an increase in sCr to ≥1.5 times baseline within the prior 7 days. Consistent with recent studies [1, 2] we also examined the effect of a 25% threshold. Categorical data were compared using the Chi-square test with Yate's correction. To adjudicate whether elevation of pCysC or sCr accompanied kidney damage, urinary KIM-1 and clusterin were evaluated according to AKI category, and relative increases of each were correlated (Pearson's method) with relative increases in sCr or pCysC. Temporal changes in variables were evaluated using repeated measures (RM) analysis of variance (ANOVA) and Sidak tests adjusted for multiple comparisons. Differences between groups were examined by ANOVA and multiplicity adjusted Sidak tests. Where appropriate values were log-transformed to normalise the distribution of values.

Table 1. Baseline Characteristics of Enrolled Subjects Receiving Cisplatin-Based Chemotherapy. ECOG: Eastern Cooperative Oncology Group Performance Status; IQR: interquartile range, a: Creatinine-based: Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) 2009 equation for creatinine equation [24], b: Cystatin C based. CKD-EPI 2012 equation for cystatin C [25]

Characteristic	*n = 27*
Male, n [%]	20 [75]
Age, median [IQR]	55 [53 -70]
Hypertension, n [%]	7 [26]
Weight kg, median [IQR]	76 [66 – 89]
Height m, median [IQR]	1.73 [1.62 – 1.78]
BMI kg/m², median [IQR]	1.93 [1.66 – 2.01]
Ischaemic Heart Disease, n [%]	2 [7]
ECOG Score , n [%]	
0	26 [96]
1	1 [4]
Cancer location, n [%]	
Oral/ pharyngeal	9 [33]
Oesophagus	13 [48]
Stomach	1 [4]
Lung	4 [15]
Cancer type, n [%]	
Squamous cell carcinoma	19 [70]
Adenocarcinoma	8 [30]
Chemotherapy, n [%]	
Cisplatin	10 [37]
Epirubicin/cisplatin/5-flurouracil	10 [37]
Epirubicin /cisplatin/capecitabine	3 [11]
Cisplatin/pemetrexed	4 [15]
Cisplatin dose	
mg/m², median [IQR]	80 [75 - 100]
Concurrent Radiotherapy, n [%]	21 [78]
Baseline eGFR	
[mL/min/1.73m²], mean ± SD	
Creatinine-based (a)	98.3 ± 17
> 60mL/min/1.73m², n [%]	27 [100]
Cystatin C based (b)	91.0 ± 29
> 60mL/min/1.73m², n [%]	27 [100]

Results

Clinical Study

Twenty-seven (27) patients were recruited. Baseline characteristics are shown in Table 1. The majority of patients were male and most were treated with concurrent radiotherapy, but none had radiotherapy to the renal bed. Mean baseline eGFRs were comparable using sCr-based and pCysC-based equations [98 vs. 91, bias: 7 (95% CI: -5 to 20) mL/min/1.73m², p = 0.07]. No patient had oliguria.

Circulating Biomarkers

Baseline mean sCr and pCysC values decreased over the first day, increased between 3d and 7d and decreased again by 14d (Fig. 1). The peak pCysC tended to occur either earlier than sCr or at the same time. To allow for this difference in timing, the maximal relative increases from baseline values in pCysC and sCr were compared.

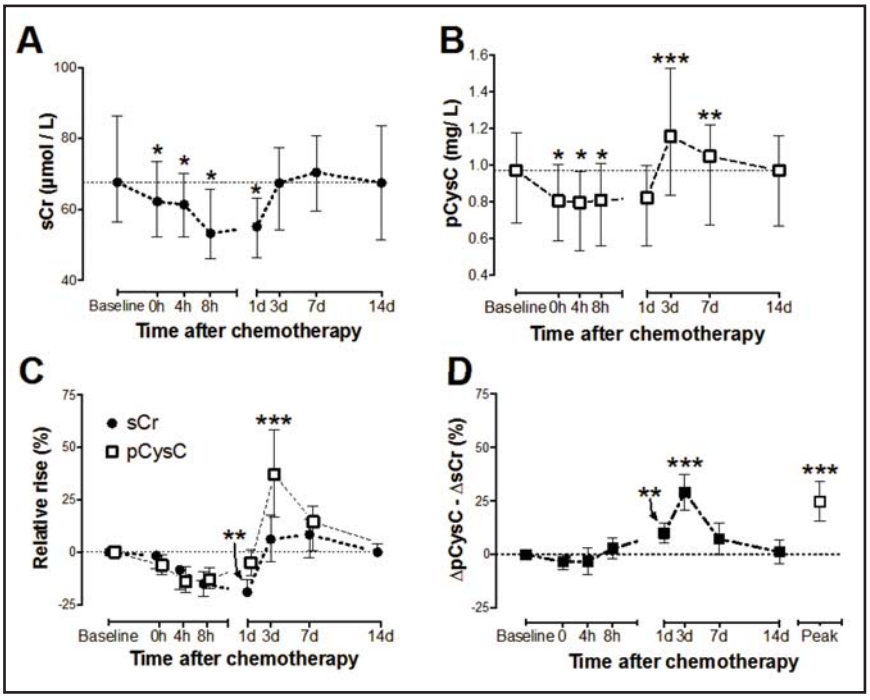

Fig. 1. sCr and pCysC After Chemotherapy in Human Subjects. A – B: sCr (A) and pCysC (B) at each study time point; C: Relative increase in sCr (closed circles) and relative increase in pCysC (open squares) compared to baseline. D: The paired difference between relative increase in pCysC (ΔpCysC) and the relative increase in sCr (ΔsCr) at each timepoint. A-C: Data expressed in median ± IQR; D: Peak: the paired difference between the maximal relative increase for sCr at any time and the maximal relative increase for pCys at any time. Data expressed in mean difference ± 95% CI; *: p <0.05, **: p < 0.01, ***: p < 0.001 for difference (A-B) vs baseline or (C-D) between sCr and pCysC.

The mean peak relative increase in pCysC was 41% compared with 16% for sCr [mean paired difference 25% (95% CI: 16% to 34%, p < 0.001)]; the relative increase was greater for pCysC in 25/27 patients (93%, p <0.001).

Only 2 (7%) patients had a ≥ 50% increase in sCr whereas nine patients (33%) had a ≥ 50% increase in pCysC. The difference in this proportion was 26% (95% CI: 9–44%), p = 0.04. Specifically, no patient had AKI-sCr, i.e. all patients who had a ≥ 50% increase in sCr also had a ≥ 50% increase in pCysC (Fig 2b, Table 2).

Table 2. Cross Tabulation of Increases in pCysC and sCr Concentrations. Data expressed in n (%)

		pCysC		
Increase from Baseline		< 50%	≥ 50%	Total
sCr	< 50%	*No-AKI* 18 (67)	*AKI-pCysC* 7 (26)	25 (93)
	≥ 50%	*AKI-sCr* 0	*AKI-Cr/CysC* 2 (7)	2 (7)
	Total	18 (67)	9 (33)	27 (100)
		pCysC		
Increase from Baseline		< 25%	≥ 25%	Total
sCr	< 25%	7 (25)	16 (59)	23 (85)
	≥ 25%	1 (4)	3 (11)	4 (15)
	Total	8 (30)	19 (70)	27 (100)

Similarly, 4 (15%) patients had a ≥ 25% increase in sCr compared with 19 (70%) who had a ≥ 25% increase in pCysC [RR: 4.8, 95% CI: 1.9-18.9, p < 0.001]. Only one patient who had a ≥ 25% increase in sCr did not have a parallel ≥ 25% increase in pCysC.

Serum uric acid concentrations were modestly reduced at 1d compared to baseline [median: 270 (IQR: 199–293) vs 283 (252–394) µmol/L, p = 0.03] but were not significantly different from baseline at any other time.

Urinary Biomarkers

Overall, compared to baseline, absolute concentrations of urinary KIM-1 and clusterin decreased over the first 16 hours, before increasing between 3d and 14d (p < 0.05 at ≥

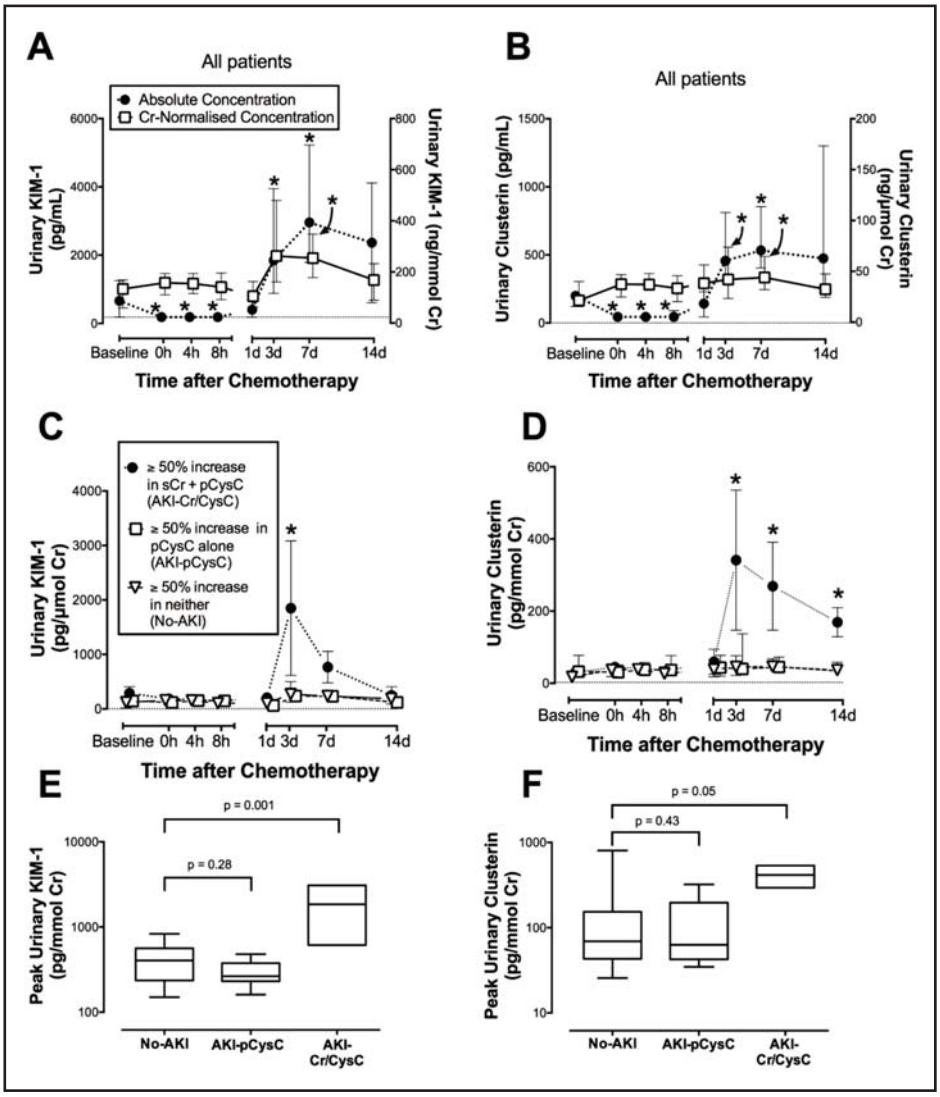

Fig. 2. Urinary Injury Biomarkers KIM-1 and Clusterin in Human Subjects Following Cisplatin-Based Chemotherapy. A and B: KIM-1 (A) and clusterin (B) over time in all subjects. C and D: Urinary KIM-1 (C) and clusterin (D) stratified for changes in sCr and pCysC. E and F. Peak urinary KIM-1 (E) and clusterin (F) stratified for changes in sCr and pCysC. A and B: Absolute concentration (closed circles) and creatinine-normalised concentration (open squares). Data presented as median ± interquartile range. *: $p < 0.05$ vs Baseline. C and D: Urinary KIM-1 (C) and clusterin (D) stratified for changes in sCr and pCysC. Patients with a ≥ 50% increase in sCr and pCysC (AKI-Cr/CysC), closed circles dotted line); patients with a ≥ 50% increase in pCysC without a corresponding ≥ 50% increase in sCr (AKI-pCysC, open square, dashed lines); or patients with a ≥ 50% increase in neither sCr nor pCysC (No-AKI, open triangles, solid line). Data presented as median ± interquartile range. *: $p < 0.05$ vs No-AKI patients. E and F: Boxes represent median, 25th and 75th centiles, and whiskers the minimum and maximum values for each group.

one time for each, Fig. 2, Panels A-B). Normalisation to urinary creatinine concentration eliminated the early decrease but not the subsequent increase in biomarker concentrations.

Urinary clusterin and KIM-1 were stratified for patients with AKI-sCr/CysC, AKI-pCysC, or No-AKI (Fig. 2, Panels C-D). At 3d, KIM-1 was increased in patients with AKI-sCr compared to No-AKI patients [median: 169 (IQR: 129–209) vs 38 (27–51) ng/mmol Cr, $p = 0.04$; Fig. 2C]. By contrast, KIM-1 was not increased at any point in patients with AKI-pCysC compared to No-AKI patients ($p > 0.05$ for all; Fig. 2, Panel C).

Similarly, clusterin was increased at 3d in AKI-sCr patients compared to No-AKI patients [median: 341 (IQR: 147–535) vs 44 (22–72) ng/mmol Cr, $p = 0.04$] and at 7d and 14d (Fig. 2,

Panel D). Clusterin was not increased at any point in AKI-pCysC patients compared to No-AKI patients. Peak KIM-1 was greater in AKI-sCr/CysC patients than No-AKI patients [median: 1849 (IQR: 614–3084) vs 404 (236–563) ng/mmol Cr, p = 0.04]. Peak KIM-1 was similar in AKI-pCysC patients and No-AKI patients [median: 265 (IQR: 231–376) vs 404 (236–563) ng/mmol Cr (p = 0.19) (Fig. 2, Panel E). There was a similar pattern for clusterin however the p-value for the differences between groups did not reach $p < 0.05$ (Fig. 2, Panel F).

There was a correlation between the peak relative increase in sCr and both the peak relative increase in KIM-1 (r = 0.43, p = 0.03), and the peak relative increase in clusterin (r = 0.41, p = 0.04). There was no correlation between the peak relative increase in pCysC and the peak relative change in KIM-1 (r = 0.29, p = 0.15) or the peak relative increase in clusterin (r = 0.15, p = 0.47).

Animal Study

Rats in the dexamethasone and control groups gained weight to a similar extent to colony animals and controls, suggesting minimal stress. In saline controls there were no significant temporal changes in any other parameters measured. No histological injury was detected in dexamethasone or saline treated rats.

Animals treated with cisplatin plus saline, and cisplatin plus dexamethasone showed an expected pattern of cisplatin-induced AKI marked by diminished weight gain, polyuria in a biphasic pattern with peaks at d2 and d10, and histological injury [4, 26]. There was no difference in these parameters - including histological injury -between the groups (data not shown).

Serum Markers

sCr was not significantly elevated in the any of the dexamethasone groups at any time (Fig. 3). Dexamethasone administration alone produced increases in pCysC in a dose-dependent manner (Fig. 4.3: B, D and F). pCysC was not significantly elevated in 0.5mg/kg dexamethasone groups at any time, however in the 2.5 mg/kg dexamethasone group pCysC was increased from 1.5 ± 0.2 mg/L (mean, SD) at baseline to 1.9 ± 0.2 mg/L at d3 (p = 0.03). In the 10 mg/kg dexamethasone group pCysC was increased from 1.4 ± 0.4 mg/L at baseline to 2.8 ± 0.6 mg/L at d1 (p = 0.04).

Administration of cisplatin 6mg/kg produced significant increases in both sCr and pCysC compared to baseline. The increase in sCr was not significantly different in either group (Fig. 3, Panel I). However, there was an augmented increase in pCysC n the cisplatin plus dexamethasone group compared with the cisplatin plus saline group [peak pCys (mean, SD): 4.4 ± 0.9 vs 3.1 ± 0.8 mg/L, p = 0.04] (Fig. 3, Panel J).

Urinary Volume and Biomarkers

Compared to baseline, a significant decrease ($p < 0.05$) in urine creatinine concentration was seen in all dexamethasone groups at d1 (Fig. 4, Panel A). Although urine volume was generally increased at day 1 after dexamethasone in all groups the difference was only significant in the 2.5mg group (Fig. 4, Panel B).

Neither the absolute, nor creatinine-normalised, concentration of KIM-1 or clusterin was significantly increased or decreased in any dexamethasone group at any time (Fig. 4, Panels C-H). For animals in the cisplatin plus saline group there was significant increase in both KIM-1 and clusterin (Fig. 4, Panel I-J). Comparing the cisplatin plus saline and the cisplatin plus dexamethasone groups, the peak concentration of KIM-1 was similar in both groups (mean, SD: 2296 ±1507 versus 1906 ± 784 ng/mmol Cr respectively, p = 0.45), as was the peak concentration of clusterin (mean, SD: 199.4 ± 216.5 versus 216.6 ± 90.20 ng/mmol Cr respectively, p = 0.45).

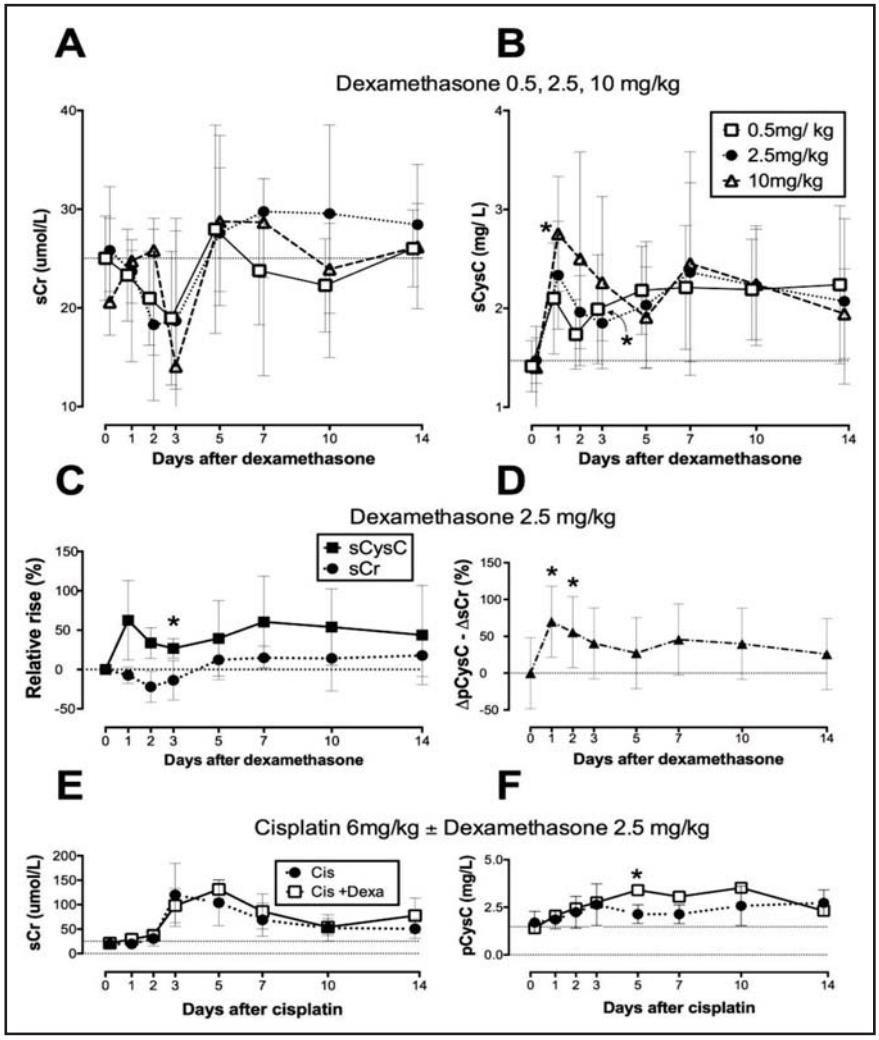

Fig. 3. sCr and pCysC after Dexamethasone Treatment in Rats. A: sCr and pCysC at each study time point in rats given dexamethasone 0.5 mg/kg (open squares), 2.5 mg/kg (closed circles) or 10 mg/kg (open triangles) intraperitoneal single dose. C: Relative change in pCysC (squares) and sCr (circles) in rats given 2.5mg dexamethasone, shown for further illustration. D: The mean paired difference (and 95% confidence interval) between relative rise in pCysC (ΔpCysC) and the relative rise in sCr (ΔsCr.) in rats given 2.5mg dexamethasone E-F: sCr (E) and pCysC (F) at each study time point in rats given cisplatin plus saline (Cis, closed circles, dotted lines) or cisplatin plus dexamethasone (Cis + Dexa).*: p <0.05 for difference versus baseline (A-B), between sCr and pCysC (G) or groups (I-J).

Discussion

In this prospective observational study of 27 patients treated with cisplatin-based chemotherapy, concurrent hydration and anti-emetics, which included dexamethasone, we observed a significantly greater increase in pCysC than sCr. Using a threshold increase of 50% to diagnose AKI [1, 2], approximately 27% more patients would be diagnosed with AKI using pCysC than sCr. However, unlike increases in sCr, increases in pCysC were not associated with increases in urinary kidney-damage biomarkers. In a subsequent animal study, treatment with 2.5mg/kg or 10mg/kg dexamethasone – or dexamethasone 2.5mg given in combination with cisplatin - significantly increased sCysC concentrations without producing - or augmenting - histological damage, changes to sCr, or urinary biomarker concentrations. These data suggest that the exaggerated increase in pCysC seen in the clinical study was independent of kidney damage, and was an effect of dexamethasone treatment. Therefore, the use of pCysC in this context would over-diagnose functional AKI. To our knowledge, this is the first report that a dexamethasone-induced increase in pCysC may confound the diagnosis of AKI in routine clinical practice.

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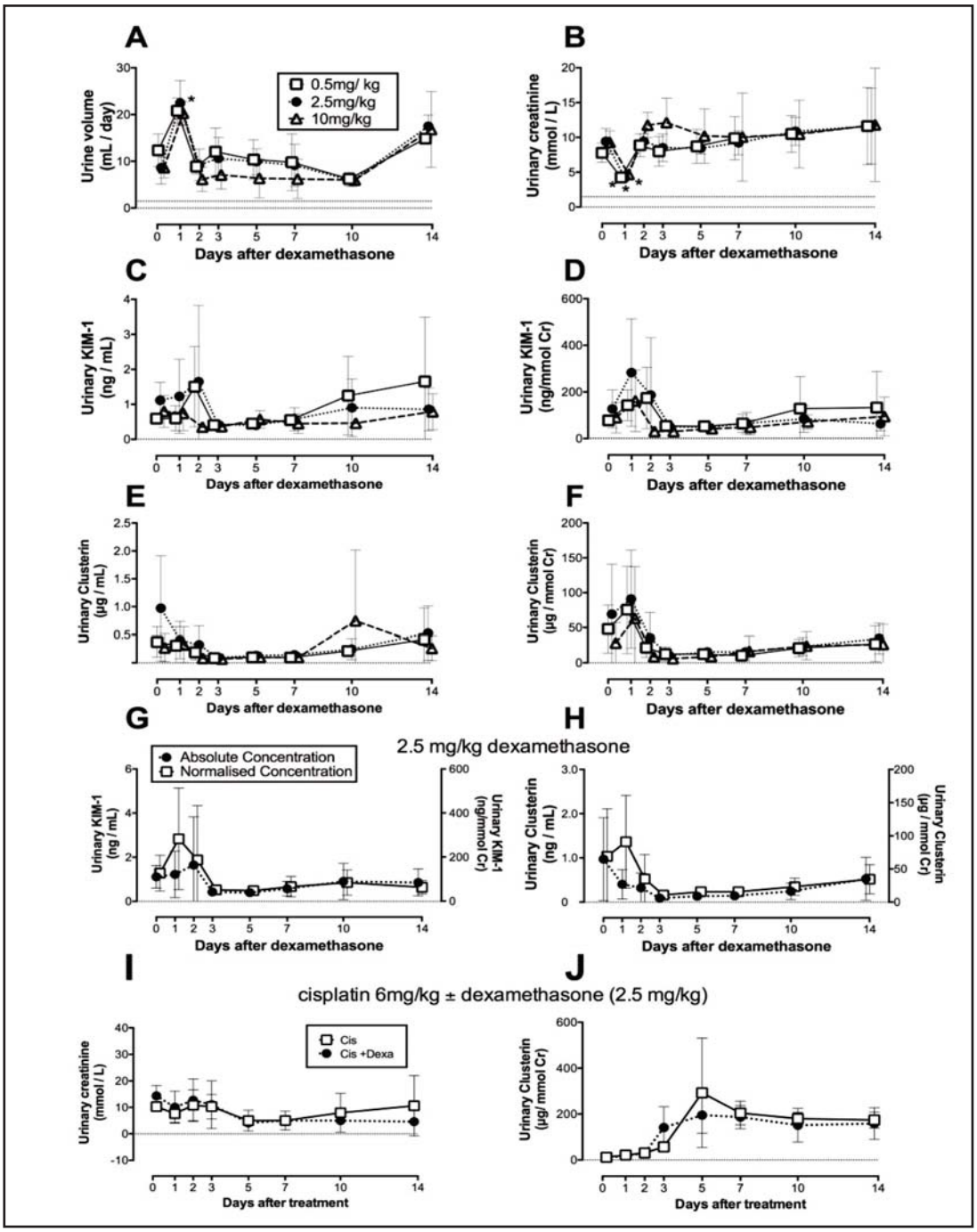

Fig. 4. Urinary Biomarkers after Dexamethasone Treatment in Rats, A-F: A: Urinary creatinine concentration; B: Urine volume; C-D: Urinary KIM-1, absolute concentration (C) and creatinine-normalised concentration (D); E-F: clusterin, absolute concentration (E) and normalised concentration (F). Animals received dexamethasone 0.5 mg/kg (open squares, lines), 2.5 mg/kg (closed circles, dots), dexamethasone 10 mg/kg (triangles, dashes) intraperitoneally as a single dose on day 0. G - H: the absolute concentration (closed circles) and normalised concentration of KIM-1 (G) and clusterin (H) in animals given 2.5mg/kg dexamethasone is shown for further illustration. I-J: KIM-1 (I) and clusterin (J) at each study time point in rats given cisplatin plus saline (Cis, closed circles, dotted lines) or cisplatin plus dexamethasone (Cis + Dexa). Data presented as mean ± standard deviation, offset on x-axis to minimise overlap. *: $p < 0.05$ for difference versus baseline (A-F), between sCr and pCysC (G) or groups (I-J).

Both pCysC and sCr vary inversely with GFR under normal conditions. CysC has approximately one-third the volume of distribution (V_d) of creatinine, and should reach a new steady state three times more rapidly following a step change in GFR [9]. Thus in a previous AKI study, more patients reached a 50% elevation of serum CysC before sCr than *vice versa* [5], and in this clinical study pCysC decreased earlier following hydration and subsequently increased earlier than sCr. However if extrarenal clearance is ignored, both pCysC and sCr should eventually change to the same relative extent following a decrease in GFR [9], with the slower change for sCr being a function of its larger V_d. If extrarenal clearance is considered, the biomarker with greater extrarenal clearance can be predicted to increase to a lesser extent [27]. Estimates of extra-renal clearance are generally greater for CysC than for creatinine, with upper estimates of approximately 21 mL/min/1.73m^2 [28] and 10 mL/min/1.73m^2 [29] respectively. Thus the relative increase in pCysC can be predicted to be less than for sCr. This might explain one study in which fewer patients had 25%, 50% or 100% elevations of sCysC than sCr [30]. However, our paired analysis demonstrated that pCysC increased more than sCr in over 90% of patients, which is contrary to expectation and suggests an effect other than renal clearance influenced either sCr or pCysC.

To adjudicate whether changes in sCr or pCysC in the clinical study reflected loss of GFR due to direct kidney injury, we examined the association between increases in sCr or pCysC and urinary kidney-damage biomarkers in the human study, and to both histological injury and kidney-damage biomarkers in the animal study. Both rodent and clinical studies have reported urinary biomarkers to be sensitive markers of cisplatin-induced AKI [19, 22]. KIM-1 is an epithelial cell receptor that enables proximal tubule cells to recognize and phagocytise apoptotic cells [19, 22]. Clusterin appears to be anti-apoptotic and may help maintain intercellular and cell-matrix interactions [21, 31]. In the clinical study increases in sCr and the incidence of AKI were modest, which may reflect the absence of high-risk patients and the effectiveness of hydration in preventing AKI. However, unlike increases in pCysC, the modest increase in sCr correlated with the relative increases in both KIM-1 and clusterin, Similarly, patients with AKI-sCr, but not AKI-pCysC had increased concentrations of KIM-1 and clusterin. These observations suggest that the increase in pCysC observed was independent of kidney injury.

We postulated that the different extent of increases in sCr and pCysC in the clinical study was because dexamethasone co-administration increased the production of pCysC disproportionately to any decrease in GFR or kidney damage. *In vitro* evidence suggests that corticosteroids increase promoter-mediated transcription of the CysC gene in malignant and non-malignant cells [18,32]. Longitudinal studies in adults have demonstrated increased pCysC values with chronic or incident corticosteroid use discordant with sCr or measured GFR [8,14,16]. In acute settings renal transplant recipients treated with methylprednisolone for acute rejection experienced a decrease in sCr but increase in pCysC [12] and in other settings adults treated with corticosteroids have experienced an acute rise of pCysC either in association with stable sCr, or a smaller increase in sCr than pCysC [11, 13, 33] as seen in our study. However, such studies have not previously examined the effect of corticosteroids on the incidence of AKI using standardised criteria.

The effect of corticosteroids on urinary biomarkers is little studied although clinical elevation of KIM-1 after radiocontrast exposure is potentially ameliorated by prednisolone [34]. *In vitro*, corticosteroids have had little effect on clusterin expression in numerous tissues including kidney-derived epithelium [35-37]. In our animal study, urinary concentrations of KIM-1 and clusterin were not significantly changed following treatment with dexamethasone, and dexamethasone did not significantly augment or ameliorate the effect of cisplatin. This accords with the unchanged levels of sCr and lack of histological injury in these animals at all doses of dexamethasone. Furthermore, cisplatin produced an expected increase in pCysC, sCr and urinary biomarkers KIM-1 and clusterin. The increase in CysC was augmented in rats concurrently given dexamethasone, but similar changes were not seen in histology (the gold standard for kidney injury in rodent studies [26]), sCr or the urinary biomarkers.

Together, these data suggest the effect of dexamethasone on pCysC is independent of kidney injury and of a magnitude that may influence the accurate diagnosis of AKI in routine clinical practice. While other corticosteroids were not evaluated, the discussed literature suggests this observation extends to other corticosteroids.

An independent effect of cisplatin, of the other anti-emetics or cytotoxic therapy, or of malignancy itself on pCysC or sCr cannot be excluded but seem less likely than the influence of dexamethasone to explain the difference observed in the clinical study. It is known that suppression of creatinine production can follow cachexia and muscle wasting due to dysphagia, nausea or malignancy; to minimise this effect, we examined only the first cycle of chemotherapy. In our study baseline eGFR was similar with sCr and pCysC, suggesting minimal baseline effect on either marker due to the underlying malignancy. *In vitro*, CysC secretion is not affected by cisplatin [18], but the effect of other anti-emetics on CysC production or release is unknown. *In vitro,* CysC transcription is increased in numerous cancer cell lines [38, 39], and in some patients with malignancy there is increased pCysC [40, 41], which has generally correlated with burden of disease [42, 43].

Limitations of the clinical study include a modest sample size, confinement to a single centre, which limit generalization of the results, enrolment of a very heterogeneous group of patients with various cancers treated by different protocols of chemotherapy, and the absence of follow-up to assess development of chronic kidney disease. Similar studies in chronic kidney disease routinely compare novel biomarkers to a directly measured (m)GFR [44]. While the absence of such comparison is a potential limitation of this study, mGFR techniques are not validated for non-steady state conditions [9] and are difficult to repeat.

Future studies should consider "real-time GFR" measurement as proposed in large-animal models [45]. While KIM-1 and clusterin have been qualified by multiple regulatory agencies for the detection of kidney toxicity in pre-clinical studies [21, 22], validation in human studies remains underway [31] and assay standardization should follow as it has with sCr and other markers. Real-time GFR, alternative markers of function, or urinary biomarkers of damage may be useful to overcome the well documented flaws of sCr and the weaknesses of pCysC demonstrated in this study, but also require further evaluation and validation [2, 9, 31].

The animal study did not evaluate fluid loading or other antiemetics and there was no significant elevation of sCysC following dexamethasone at an allometric approximation (0.5 mg/kg) of the dose used in patients. However, the estimate of equivalent drug doses across species and using different routes of delivery is inexact [33], and as shown in Fig. 3B the direction of effect of 0.5mg/kg dexamethasone was at all times consistent with that seen in the clinical study and for greater dexamethasone doses.

While the practice of normalising urinary kidney damage biomarkers to creatinine concentration remains under intense discussion [23, 46, 47], our clinical data, particularly over the first day where the absolute concentrations of urinary creatinine and biomarkers were reduced in association with diuresis, helped justify our decision to analyse creatinine-normalised concentrations. A similar effect was observed with use of dexamethasone alone in the animal study.

Conclusion

These results suggest that in the context of dexamethasone co-administration, pCysC is increased independent of kidney injury, does not improve on sCr, and may be unreliable in the diagnosis of AKI.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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