# Kidney damage biomarkers detect acute kidney injury but only functional markers predict mortality after paraquat ingestion.

Fahim Mohamed [a,b,c,d,e], Nicholas A. Buckley [a, b, e], Shaluka Jayamanne [a], John W. Pickering [f,g], Philip Peake [d], Chathura Palangasinghe [a],Thilini Wijerathna [a], Indira Ratnayake [a], Fathima Shihana [a], Zoltan H. Endre [d,g]*

(a) South Asian Clinical Toxicology Research Collaboration, University of Peradeniya, Peradeniya, Sri Lanka

(b) Clinical Pharmacology and Toxicology Group, Professorial Medicine Unit, The Prince of Wales Clinical School, University of New South Wales, NSW, Australia

(c) Department of Pharmacy, Faculty of Allied Health Sciences, University of Peradeniya, Sri Lanka

(d) Department of Nephrology, Prince Of Wales Hospital and Clinical School, University of New South Wales, Sydney, Australia

(e) Department of Pharmacology, SOMS, Sydney Medical School, University of Sydney, NSW, Australia

(f) Department of Medicine, University of Otago, Christchurch, New Zealand

(g) Emergency Department, Christchurch Hospital, Christchurch, New Zealand

* Author for correspondence and reprints:

Professor Zoltan H. Endre

Department of Nephrology, Prince of Wales Hospital,

High Street, Randwick, Sydney, NSW 2031, Australia

Phone: +61293824418, Fax: +61293824409

Email: z.endre@unsw.edu.au

**E-mail addresses;**

Fahim Mohamed; fahim.cader@gmail.com

Nicholas A. Buckley; nicholas.buckley@sydney.edu.au

Shaluka Jayamanne; shalukajaya@yahoo.com

John W. Pickering; john.pickering@icloud.com

Philip Peake: phyllip.peake@sesiahs.health.nsw.gov.au

Chathura Palangasinghe; chathura7@yahoo.com

Thilini Wijerathna; thilini.wijerathna@gmail.com

Indira Ratnayake; rmiu.ratnayake@yahoo.com

Fathima Shihana; fathimashihana@gmail.com

Zoltan H. Endre; z.endre@unsw.edu.au

**Key words;**

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**Abstract**

Acute kidney injury (AKI) is common following paraquat ingestion. The diagnostic performance of injury biomarkers was investigated in serial blood and urine samples from patients from 5 Sri Lankan hospitals. Functional AKI was diagnosed using serum Creatinine (sCr) or serum cystatin C (sCysC). The 95$^{th}$ centile in healthy subjects defined the urinary biomarker cutoffs for diagnosing structural AKI. 50 poisoned patients provided 2 or more specimens, 76% developed functional AKI [AKIN stage 1 (n=12), 2 (n=7) or 3 (n=19)]; 19/26 patients with AKIN stage 2/3 also had functional AKI by sCysC criteria (≥50% increase). Urinary cystatin C (uCysC), clusterin (uClu) and NGAL (uNGAL) increased within 24 hours of ingestion compared with NoAKI patients and healthy controls. Each biomarker demonstrated moderate diagnostic utility [AUC-ROC: uCysC 0.79, uNGAL 0.79, uClu 0.68] for diagnosis of functional AKI at 16 hours. Death occurred only in subjects with functional AKI. Structural biomarker-based definitions detected more AKI than did sCr or sCysC, but did not independently predict death. Renal injury biomarkers did not add clinical value to patients who died rapidly due to multi-organ failure. Use of injury biomarkers within 16-24 hours may guide early intervention for reno-protection in less severe paraquat poisoning.

## 1. Introduction

Deliberate self-poisoning with paraquat herbicide is common and has an estimated case fatality of more than 50% (Dawson et al., 2010), particularly when followed by acute kidney injury (AKI) (Kim et al., 2009; Lee et al., 2002). Paraquat-induced oxidative stress in the acute phase leads to toxicity in many organs particularly lungs and kidneys (Dinis-Oliveira et al., 2008; Gawarammana and Buckley, 2011) while paraquat induced AKI may aggravate toxicity to other organs by decreasing paraquat clearance (Beebeejaun et al., 1971).

Several studies have shown that the rapid increase in sCr following paraquat poisoning (Gil et al., 2009; Roberts et al., 2011) cannot be solely driven by the AKI-mediated decrease in glomerular filtration rate (GFR) and hence over-estimates true renal functional loss (Fahim et al., 2013). Therefore, alternative approaches would be useful for early diagnosis or confirmation of paraquat-induced nephrotoxicity. A panel of seven biomarkers proposed by the Predictive Safety Testing Consortium (PSTC) was qualified by the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) for safety assessment in pre-clinical drug development studies (Dieterle et al., 2010; Ferguson et al., 2008). These diagnose AKI early with high specificity and sensitivity depending upon site and mode of renal injury (Bonventre et al., 2010).

The clinical utility of most novel urinary biomarkers in detecting AKI has not been explored after paraquat poisoning. A few small clinical studies have utilised serum cystatin C (sCysC), urinary kidney injury molecule-1 (uKIM-1), plasma (pNGAL) (neutrophil gelatinase-associated lipocalin) and urinary NGAL (uNGAL) to predict death (Roberts et al., 2011) and in one study to diagnose AKI, where increases in sCr preceded increases in uNGAL and uKIM-1 (Gil et al., 2009). In contrast, urinary KIM-1, urinary cystatin C (uCysC) and albumin (uAlb) were sensitive biomarkers in predicting paraquat-induced AKI within 16-24 hours in a nephrotoxic rat model as defined by histopathological change (Wunnapuk et al., 2013). In order to determine the clinical utility of injury biomarkers, we performed frequent serial biomarker measurements in a prospective patient cohort following paraquat poisoning, utilising the FDA/EMEA qualified panel of biomarkers plus additional selected novel urinary damage biomarkers.

We hypothesized that a panel of novel urinary structural damage biomarkers are superior to serum creatinine in independently detecting paraquat-induced nephrotoxic AKI (ToxAKI) and correlate with specific pathways of renal injury. The main objective of this study is to evaluate the utility of PSTC biomarkers panel and additional selected urinary biomarkers in early diagnosis of paraquat-induced ToxAKI and to explore whether increase in specific biomarker relate to mechanism-specific injury pathways. The other aim of this study is to evaluate whether pre-clinical paraquat ToxAKI rodent model findings translate into clinical practice.

## 2. Methods

### 2.1 Study design and data collection

This nested cohort study within an ongoing multi-centre observational study on self-poisoning in Sri Lanka was approved by the human research ethics committees of both the University of New South Wales (Sydney), Australia and the University of Peradeniya (Peradeniya, Sri Lanka). Between October 2010 and March 2013, patients admitted to study hospitals within 24 hours of paraquat ingestion were consented after initial resuscitation and clinical management using written informed consent from each patient or a relative. Patients who were < 15 years, pregnant, had co-ingested other toxins, or unable to provide samples were excluded. Paraquat ingestion was confirmed by a positive urine dithionate test. Demographic and clinical data were collected from consenting patients until discharge.

### 2.2 Sample collection and biomarker assays

Blood and urine samples were collected at 4, 8, 16 and 24 hours after ingestion where possible, then daily until discharge or death and at follow-up at one and three months. Blood and urine samples were also collected from consenting healthy volunteers to establish normal baseline biomarker concentrations. All samples were processed within 30 minutes of collection. Blood samples were spun at 2000-3000 rpm and serum samples were transferred in

to small cryotubes. Urine samples were immediately centrifuged at 1500-2000 rpm and the supernatant stored. Both serum and urine aliquots were stored at -20°C for up to 3 months and then -70°C until batch analysis within 6 months.

Biomarker assays were conducted batch-wise on samples collected from both patients and healthy controls. Serum and urine creatinine were measured using the Jaffe method (kinetic method, rate blank and compensated) on a Hitachi 912 automatic analyser (Roche, Japan). Serum CysC was quantified using microparticle enhanced immunoturbidimetry on a clinical chemistry analyser (Konelab™, Thermo Fisher, Waltham, MA) following the manufacturer's recommendations.

DuoSet ELISA kits (R&D systems®) were used to assay uKIM-1, and uClu. Urinary IL-18 was measured using the platinum enzyme-linked immunosorbent assay (Bender MedSystems, Vienna, Austria). Intra- and inter-assay precision for ELISA was <10%. Six AKI biomarkers [uCysC, uAlb, urinary trefoil factor 3 (uTFF3), osteopontin (uOstP), beta-2-microglobulin (uβ2M) and uNGAL] were quantified simultaneously using Bio-Plex Pro™ RBM Human Kidney Toxicity Assays panel 2 on the Bio-Plex 200 system (BIO-RAD, USA). Inter- and intra-assay precision was <15% and <5% respectively. Serum and urinary paraquat levels were measured at the Therapeutic Research Centre, University of Queensland, Brisbane, Australia, using LC–MS/MS (Wunnapuk et al., 2011). Biomarker concentrations were reported as the absolute concentration or normalised to uCr excretion (Ralib et al., 2012; Westhuyzen et al., 2003). The area under the concentration curve (24hrAUC, a measure of the biomarker concentration integrated over time) at 24 hours for each biomarker was calculated using the trapezoidal rule. Apparent creatinine clearance was calculated in ml/min from: [urine flow rate (ml/min) * uCr (mg/dl)) / sCr].

**2.3 Outcome measurement**

Functional AKI was defined by two approaches based either on an increase in sCr or sCysC. Diagnostic performance of each urinary biomarker was assessed using both these definitions and compared in sensitivity analysis. Acute Kidney Injury Network (AKIN) criteria were used to define functional AKI based on sCr (Mehta et al., 2007) and to categorise patients into severity stages. Despite noted limitations of sCr, AKIN definition was used in this study since it is widely used definition to assess the performance of novel injury biomarkers in clinical studies (Siew et al., 2011; Waikar et al., 2012). Further, clinical biomarker studies have used different percentage change in sCr or sCysC to define functional AKI (Briguori et al., 2010; Nejat et al., 2010; Pickering and Endre, 2014; Siew et al., 2011; Waikar et al., 2012). Therefore, development of moderate or severe AKI (stage 2 or 3, an increase in sCr of ≥200% or 300% respectively) was selected as the primary outcome definition (Basu et al., 2014) of functional AKI in this cohort since

rapid increases in serum creatinine within 24 hours of paraquat ingestion does not represent true renal functional loss (Mohamed et al., 2015). Alternatively, a 50% or greater increase in sCysC (Nejat et al., 2010) was used to diagnose functional AKI. Ideally, these definitions require a baseline level obtained within the three months prior to renal injury (Bellomo et al., 2004; KDIGO, 2012), a parameter not available in any of our patients as in many other research and hospital settings (Gaiao and Cruz, 2010; Hoste et al., 2006). Therefore, we used the lowest sCr or sCysC value in survivors measured prior to hospital discharge or at follow-up (Chertow et al., 2005; Endre et al., 2010; Lopes et al., 2008) and the MDRD75 (Bellomo et al., 2004; Gaiao and Cruz, 2010; Hoste et al., 2006; KDIGO, 2012) and CKD-EPI75 equations (Inker et al., 2012) to estimate baseline sCr and sCysC in non-survivors if these were lower than the measured lowest value.

Defining AKI based on structural (damage) and/or functional biomarker concentrations has been proposed recently (Murray et al., 2014), although with different approaches to define biomarker cutoff values (Basu et al., 2014; Pickering and Endre, 2013a, b). Defining AKI based on structural biomarkers may be useful in clinical conditions where sCr based definition may not be appropriate. We defined structural-AKI when structural damage biomarker concentration exceeded the 95th centile of the biomarker concentrations in healthy volunteers from a population with similar demographic characteristics to the patients (Basu et al., 2014). Similar approaches using healthy reference cutoff values have commonly been used for diagnosis and risk stratification of myocardial injury, utilising cardiac troponin (Alpert et al., 2000; Morrow et al., 2007).

**2.4 Statistical Analysis**

Biomarker concentrations were compared with those in healthy controls to identify renal injury at each time point. The time course for each biomarker was assessed graphically. Continuous and categorical variables were summarised using median and interquartile range, or mean ± standard error of mean (SEM), and proportions respectively. The data were compared using the Wilcoxon rank sum and Kruskal-Wallis tests for continuous variables and Fisher's exact test for categorical variables. The Spearman coefficient was used to estimate the correlation between biomarkers. The diagnostic performance of each biomarker at each time point, and the peak biomarker concentration within 24 hours of ingestion were evaluated by area under the receiver operator characteristic curve (AUC-ROC) and compared with healthy subject or no-AKI patients as controls. The optimal threshold for each biomarker was selected based on the Youden index, the cutoff value with the maximum sum of specificity and sensitivity. Statistical analyses were conducted using GraphPad Prism version 6 (GraphPad Software, San Diego, USA) and STATA IC10 (StataCorp, 2007, TX, USA).

## 3 Results

### 3.1 Patient recruitment

Eighty-five patients with a history of paraquat self-poisoning were admitted to the study hospitals. Four patients declined to participate (Fig. 1). Since diagnosis of AKI using the AKIN or KDIGO criteria requires at least two sCr measurements, patients were divided into two groups based on the number of samples available: a cross-sectional and a longitudinal (main) cohort. The cross-sectional cohort (CSC) comprised patients with fewer than two blood and/or urine samples within first 7 days after paraquat ingestion (n=31). Patients who provided at least two blood and urine samples formed the main cohort (n=50) (Fig. 1).

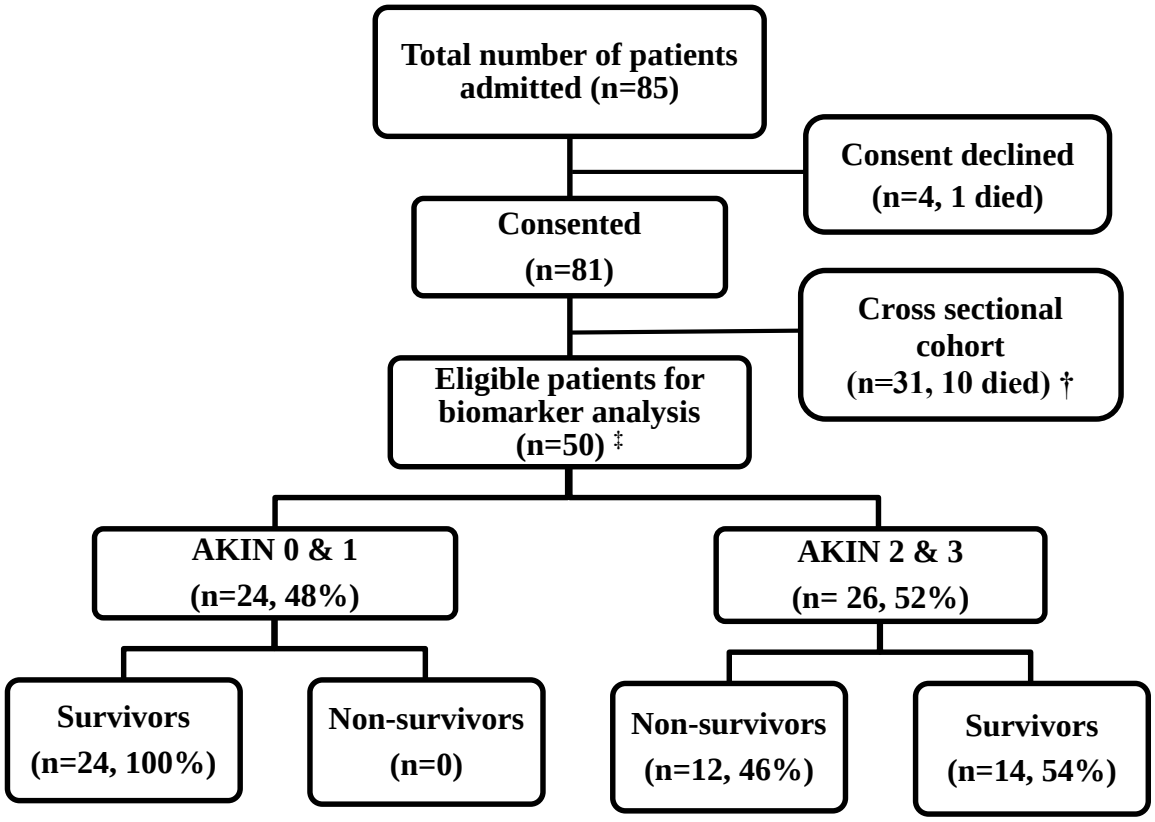



***Fig. 1. Patient recruitment profile***

*† Cross sectional cohort; these patients consented and provided only one blood and/or urine sample within first 7 days. Of 31 patients in this group, 10 patients died within 48 hours. ‡ All patients in this group provided at least two blood and urine samples and considered for the main analysis.*

### 3.2 Longitudinal (main) cohort: AKI and baseline characteristics

Twenty four patients (48%) demonstrated an increase in sCr < 100% (NoAKI=12, AKIN stage 1 =12) while 26 (52%) increased ≥100% (AKIN stage 2, n=7, or stage 3, n=19) (Fig. 1). Baseline characteristics were similar between these

two groups except for sCr and maximum 24 hour serum paraquat levels. The latter were higher ($p<0.001$) for patients with a sCr increase ≥100% (Table 1) as seen in a different paraquat poisoning cohort (Weng et al., 2012). Twelve of 26 patients who had sCr increase ≥100% died, while no patients with increase <100% died ($p=0.0001$).

***Table 1. Baseline demographic and clinical characteristics***

| **Baseline characteristics** | **No-AKI & AKIN1 (n=24)** | **AKIN 2/3 (n=26)** | **p** |
|---|---|---|---|
| Age (years) | 25 (19-34) | 25 (19-32) | 0.77 |
| Male gender (%) | 58 % | 50% | 0.74 |
| Weight (kg) | 50 (45-59) | 50 (40-58) | 0.92 |
| Amount ingested (ml) | 20 (10-45) | 20 (20-50) | 0.08 |
| Time to admission (hours) | 4 (1-6) | 3.5 (2-9) | 0.37 |
| Pulse (beats/minutes) | 88 (80-88) | 80 (76-87) | 0.21 |
| BP systolic (mm Hg) | 110 (110-120) | 115 (110-120) | 0.88 |
| BP diastolic | 70 (70-80) | 75 (70-80) | 0.93 |
| sCysC (mg/l) | 0.7 (0.6-0.8) | 0.7 (0.6-0.9) | 0.56 |
| sCr (mg/dl) | 0.73 (0.66-0.87) | 1.03 (0.55-1.40) | 0.03 |
| 24 hour maximum serum-paraquat levels (ng/ml) | 21 (10-212) | 663 (97-1430) | 0.0004 |
| Fatal outcome | 0 | 12 (46%) | 0.0001 |
| Number of patients with ≥50 % relative increase in sCysC (n) | 0 | 19 (74%) ‡ | - |

*Results are shown as median (Inter quartile range) or n (%). ‡ There were 7 patients who had a relative increase of sCr ≥ 100% but less than 50% increase in sCysC, 3 were diagnosed as AKIN stage 2 and 4 were AKIN stage 3.*

An increase of ≥50% in sCysC was seen only in patients with a relative rise in sCr of ≥100% [(n=19, 10 deaths), Table 1, Fig. 2]. Among 31 patients (death, n=2) with <50% increase in sCysC, 19 had functional AKI by sCr definition. Increases in sCr occurred earlier in these patients (within 8 hours) compared to sCysC (by 16 hours) (Fig. 2a). In contrast, when lesser increases in both functional biomarkers were observed (sCr <100% and sCysC <50%), both analytes were increased by 16 hours (Fig. 2b).

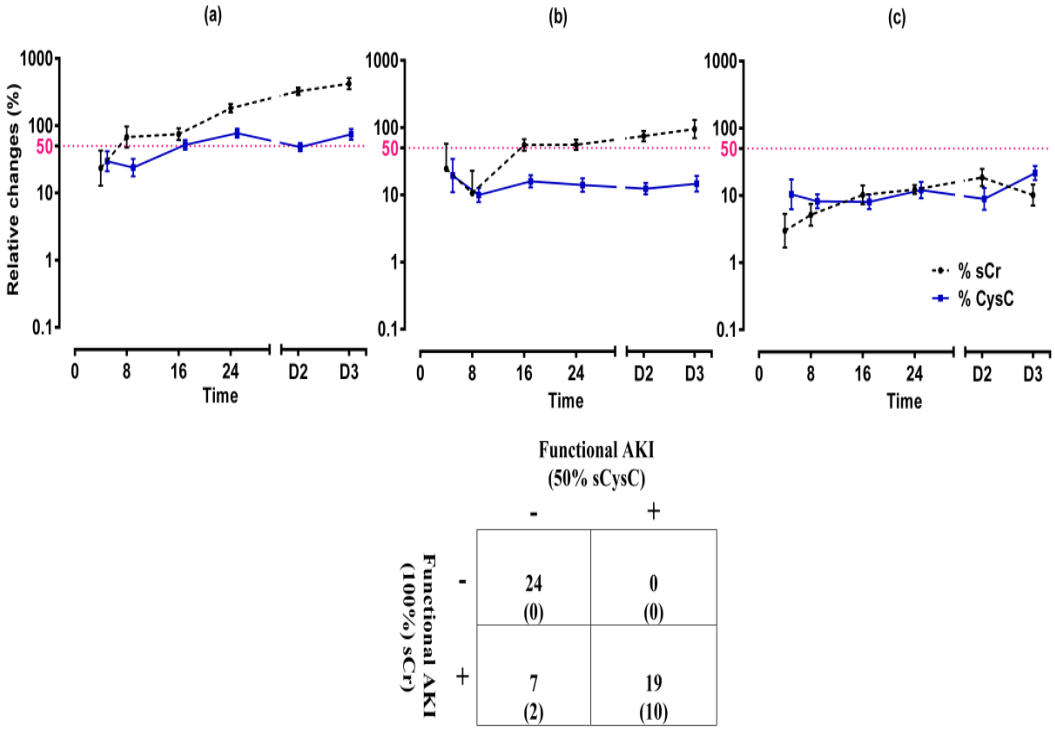



***Fig. 2. Temporal profile of relative changes in sCr and sCysC in paraquat poisoning***

*Relative changes (mean ± SEM) of sCr (black circles) and sCysC (blue squares) are shown. Relative changes in sCr occurred earlier than sCysC when a relative change in sCr was > 50%. Serum Cr continued to increase in severe poisoning while sCysC peaked between 24-48 hours and then reached a new steady state. Panel (a) sCr ≥100% & sCysC≥50%; (b) sCr≥50 % but < 100% and sCysC<50% (c) both sCr and sCysC <50%*

### 3.3 Biomarkers in the cross sectional and main cohort

Biomarker concentrations measured at 16 hours in the main cohort and maximum biomarker concentrations in patients from the cross sectional cohort [median age 28 (IQR 19-44) years] are shown in Supplementary Fig1. Although biomarker concentrations in most cross sectional cohort patients were similar to patients in the main cohort, very elevated levels were seen in some non-survivors.

### 3.4 Urinary biomarker profiles in the main cohort

Both absolute and normalised concentrations of uCysC, uClu and uNGAL increased to above the 75$^{th}$ centile (of healthy control values) in patients who developed AKI stage 2 or 3, while concentrations remained low in patients who didn't develop AKI or had mild AKIN stage 1 (Fig. 3). Normalisation of uCysC, uClu, uNGAL and particularly uKIM-1 to uCr better separated the AKIN 2/3 group from patients who didn't develop AKI (Fig. 3). At 16-24 hours,

uCysC, uClu and uNGAL were significantly increased ($p<0.01$) compared to healthy controls or no-AKI patient controls (Fig. 3).

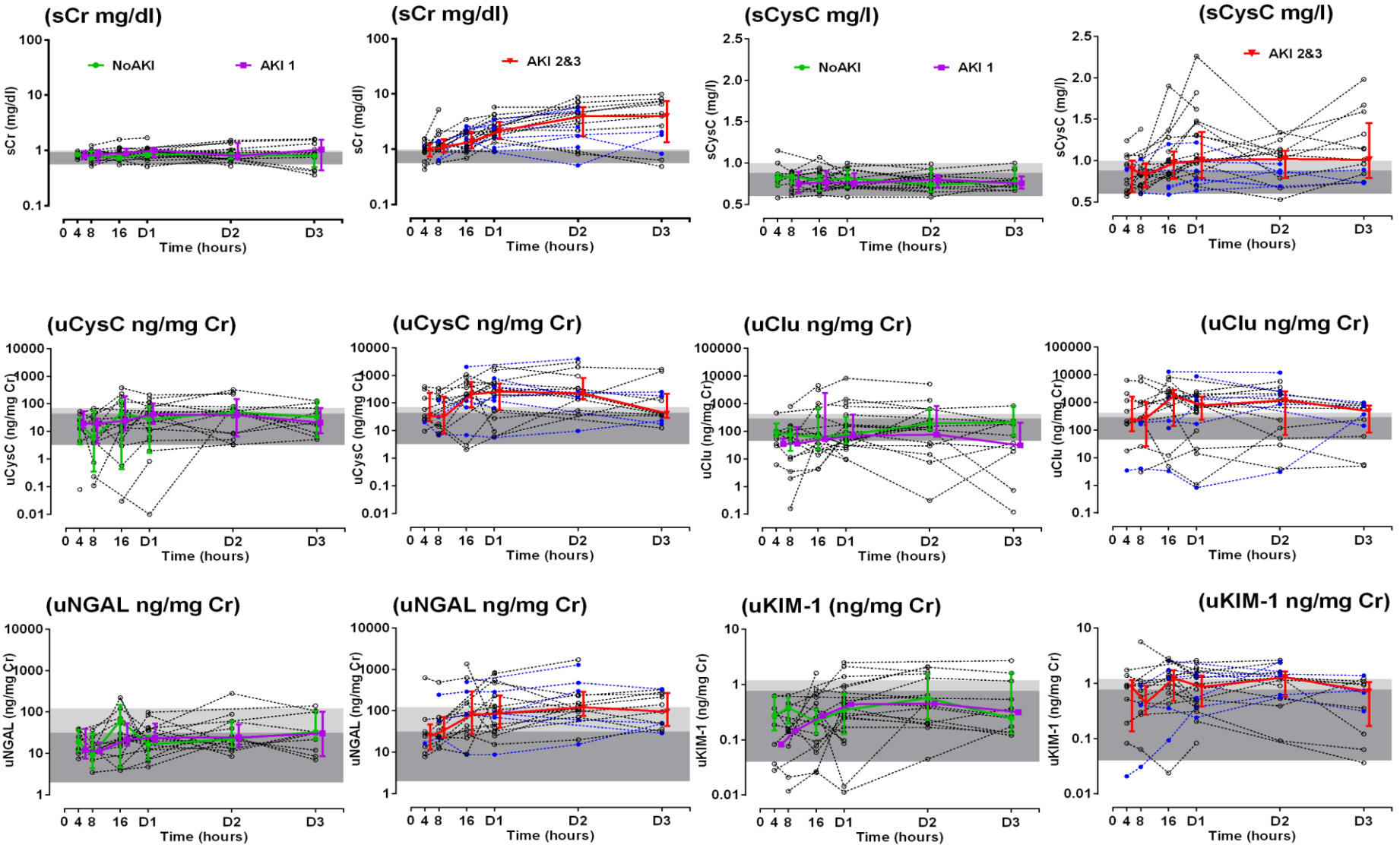



***Fig. 3. Biomarker concentration (normalised to urinary creatinine) profiles following paraquat poisoning***

*Individual patient's absolute concentrations of sCr, sCysC and normalised biomarker concentrations of uCysC, uClu, uKIM-1 and uNGAL are shown (dashed lines). Blue dashed lines depict patients who developed AKIN stage 2 and other patients in this group represent AKIN stage 3. The dark bolded line in each graph represents the median (± IQR) change in each group (green line-No-AKI group; purple line-AKIN stage 1 group, red line-AKI≥2 group). The grey shaded area illustrates the normal range based on respective biomarkers measured in healthy individuals (dark grey area-5th to 75th centiles; light grey area-75th to 95th centiles of the normal range).*

In contrast, concentrations of several other biomarkers (uAlb, uβ2M, uIL-18, uTFF3 and uOstP) were not markedly increased and did not distinguish between the two groups. Urinary albumin increased in all patients regardless of AKI (Supplementary Fig. 2). Time-course of absolute urinary biomarker concentrations are presented in Supplementary Figs 3 and 4.

### 3.5 Twenty four hour peak biomarker concentrations (24hrMax) and 24hrAUC

Both absolute and normalised 24hr peak concentrations of uCysC, uClu and uNGAL in patients who developed AKIN stage 2 or 3 were higher (normalised uCysC, $p<0.0001$, uClu, $p<0.0005$, uNGAL, $p< 0.005$) and correlated with AKI severity (Supplementary Fig. 5). The total area under the biomarker concentration curve over 24 hours (24hrAUC, 24hr total biomarker excretion which is a measure of the biomarker concentration integrated over time) for each biomarker was also higher in patients who developed AKIN 2/3 (normalised uCysC, $p<0.005$, uClu, $p<0.01$, uNGAL, $p< 0.05$). The gradients observed between the normalised urinary biomarker increases and the maximum sCr increases are a measure of the enhancement resulting from normalisation to urinary creatinine. Normalisation has enhanced urinary biomarker diagnostic performance in each case illustrated (Supplementary Fig. 5).

### 3.6 Structural and functional AKI

The time course profiles of structural damage biomarkers exhibited increase within the first 24 hours with the duration of uNGAL increase was being the briefest of these biomarkers (Fig. 4). The $95^{th}$ centile biomarker concentrations in sixty-three healthy Sri Lankan volunteers [median age 28 years (IQR: 26-33), 70% male] used to define structural-AKI, were uCysC: 70 ng/mg Cr; uClu: 420 ng/mg Cr; and uNGAL: 120 ng/mg Cr. Table 2 summarises 24 hour peak concentrations of urinary biomarkers and serum paraquat in patients with or without functional or structural AKI as per recently proposed AKI definition matrix (Murray et al., 2014). Patients who were structural biomarker positive based on uCysC or uClu definition but functional negative had similar (Table 2 a) or even higher (Table 2 b) serum paraquat concentration compare to patients who had functional AKI but no structural AKI.

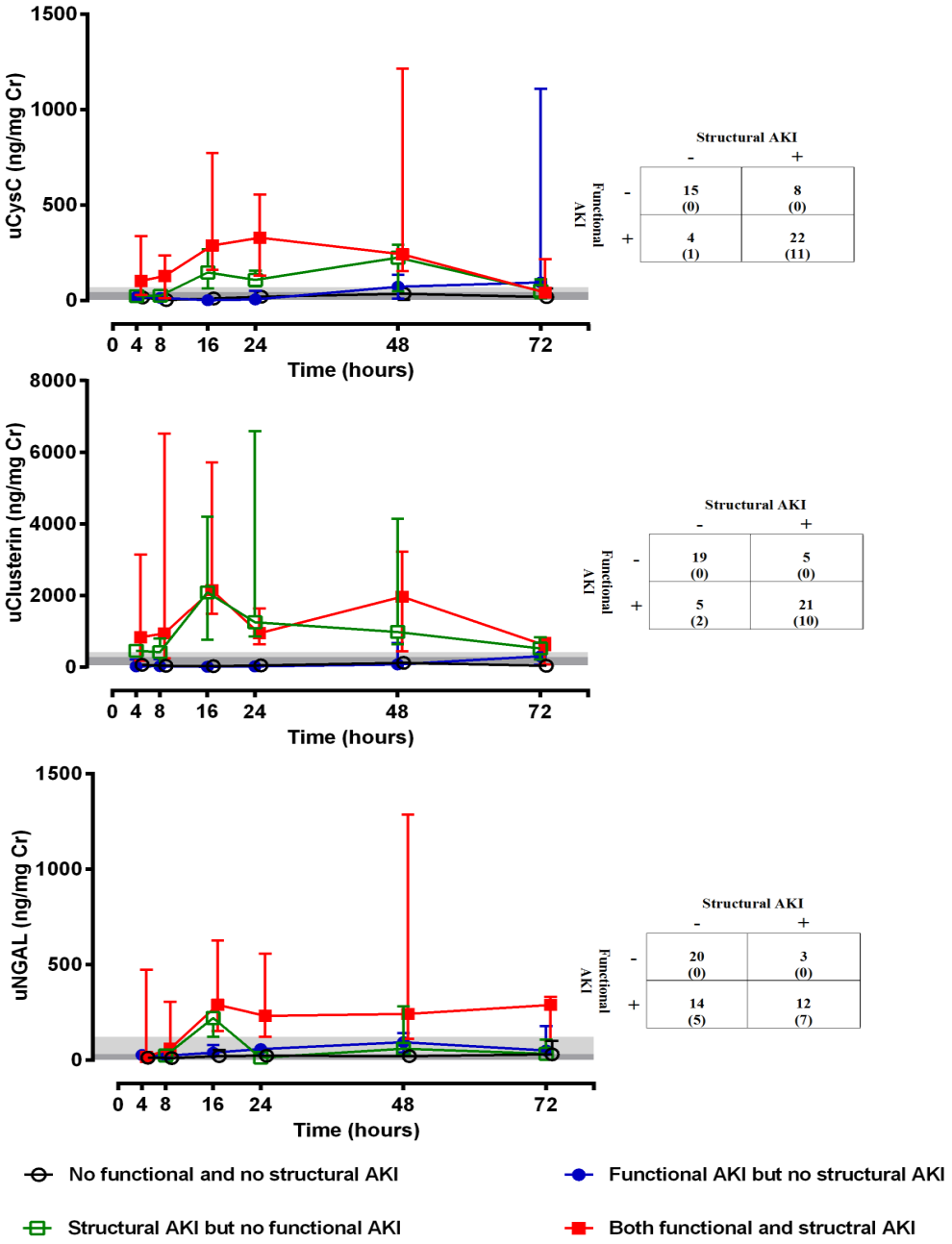



***Fig. 4 Biomarker time courses according to definition as functional or structural or combined functional and structural AKI***

*In contrast to the other structural markers, the time course of uNGAL appears brief, while those of uCysC and uClu remain increased for at least 48 hours. The lines are represented as follows; No functional and no structural AKI (black), Functional AKI but no structural AKI (blue), Structural AKI but no functional AKI (green), Both functional and structural AKI (red). Sample volume collected was not sufficient in one patient for quantifying uNGAL and uCysC using the Bioplex assays and hence only uClu was measured. Serum creatinine ≥ 100% (AKI≥2) is defined as functional AKI while biomarker concentration >95$^{th}$ centile value in healthy volunteers (uCysC: 70 ng/mg Cr; uClu: 420 ng/mg Cr; and uNGAL: 120 ng/mg Cr) were used to define structural AKI. Each cell in the matrix displays number of patients with number of deaths inside parenthesis.*

***Table 2 Peak urinary biomarker and serum creatinine concentrations in patients with or without functional and structural AKI***

(a) uCysC definition

| | No structural-AKI | Structural-AKI |
|---|---|---|
| No functional-AKI | B 26 (2-50)<br>P 13 (10-21)<br>[n=15, 31%] | B 135 (97-206)<br>P 398 (84-750)<br>[n=8, 16%] |
| Functional-AKI | B 23 (13-38)<br>P 461 (62-1076)<br>[n=4, 8%)] | B 446 (26-782)<br>P 663 (276-3700)<br>[n=22, 45%] |

(b) uClu definition

| | No structural-AKI | Structural-AKI |
|---|---|---|
| No functional-AKI | B 90 (45-160)<br>P 16 (10-22)<br>[n=19, 38%] | B 1457 (1048-4546)<br>P 574 (398-750)<br>[n=5, 10%] |
| Functional-AKI | B 94 (21-216)<br>P 90 (89-767)<br>[n=5, 10%] | B 2337(1212-6298)<br>P 992 (498-3700)<br>[n=21, 42%] |

(c) uNGAL definition

| | No structural-AKI | Structural-AKI |
|---|---|---|
| No functional-AKI | B 33 (21-48)<br>P 13 (11-116)<br>[n=20, 41%] | B 220 (122-221)<br>P 84 (21-398)<br>[n=3, 6%] |
| Functional-AKI | B 61 (32-84)<br>P 832 (276-3700)<br>[n=14, 29%] | B 390 (190-706)<br>P 555 (90-767)<br>[n=12, 24%] |

*Definitions: Functional-AKI: AKIN stage 2/3 (≥100% increase in sCr), Structural AKI: uCysC > 70 ng/mg Cr or uClu > 420 ng/mg Cr or uNGAL 120 ng/mg Cr. Normalised peak concentrations (in ng/mg Cr) were attained within 24 hours of admission. Biomarker value (first row in each cell represented as 'B') and serum paraquat (ng/ml) concentrations (second row in each cell represented as 'P') are presented as median (Inter quartile range). Number of patients under each spectrum of AKI combination is presented inside parenthesis (third row in each cell).*

Overall 65% of subjects diagnosed with structural AKI with at least one of these biomarkers (Fig. 5) while uCysC diagnosed the most patients (n=30). Structural-AKI diagnosed by uCysC, uClu and uNGAL occurred in 16% (n=8),

10% (n=5) and 6% (n=3) respectively of patients not diagnosed AKI by changes in sCr ≥100% (Table 2, Figs. 4 & 5). The combination of uClu and uCysC appeared best and diagnosed all but 3 patients who developed functional AKI (Fig. 5 b & f).

The same biomarker cutoffs were evaluated to determine the sensitivity and specificity for AKI and death (Table 3). Urinary CysC had good sensitivity for death (0.92), whereas uNGAL was poor (0.58). However, death occurred only in subjects with functional AKI [AKIN stage 2/3 (e-h) or >50% increase in sCysC (a-d)] or both functional and structural AKI (Fig. 4 & 5). Using other cutoffs ($97.5^{th}$ or $99^{th}$ centile) of normal biomarker concentrations did not influence these observations. At 24 hours, sCr revealed an excellent AUC-ROC profile [0.95 (95% CI 0.9-1] to predict death while sCysC also produced a good prognostic performance [0.8 (0.7-1)].

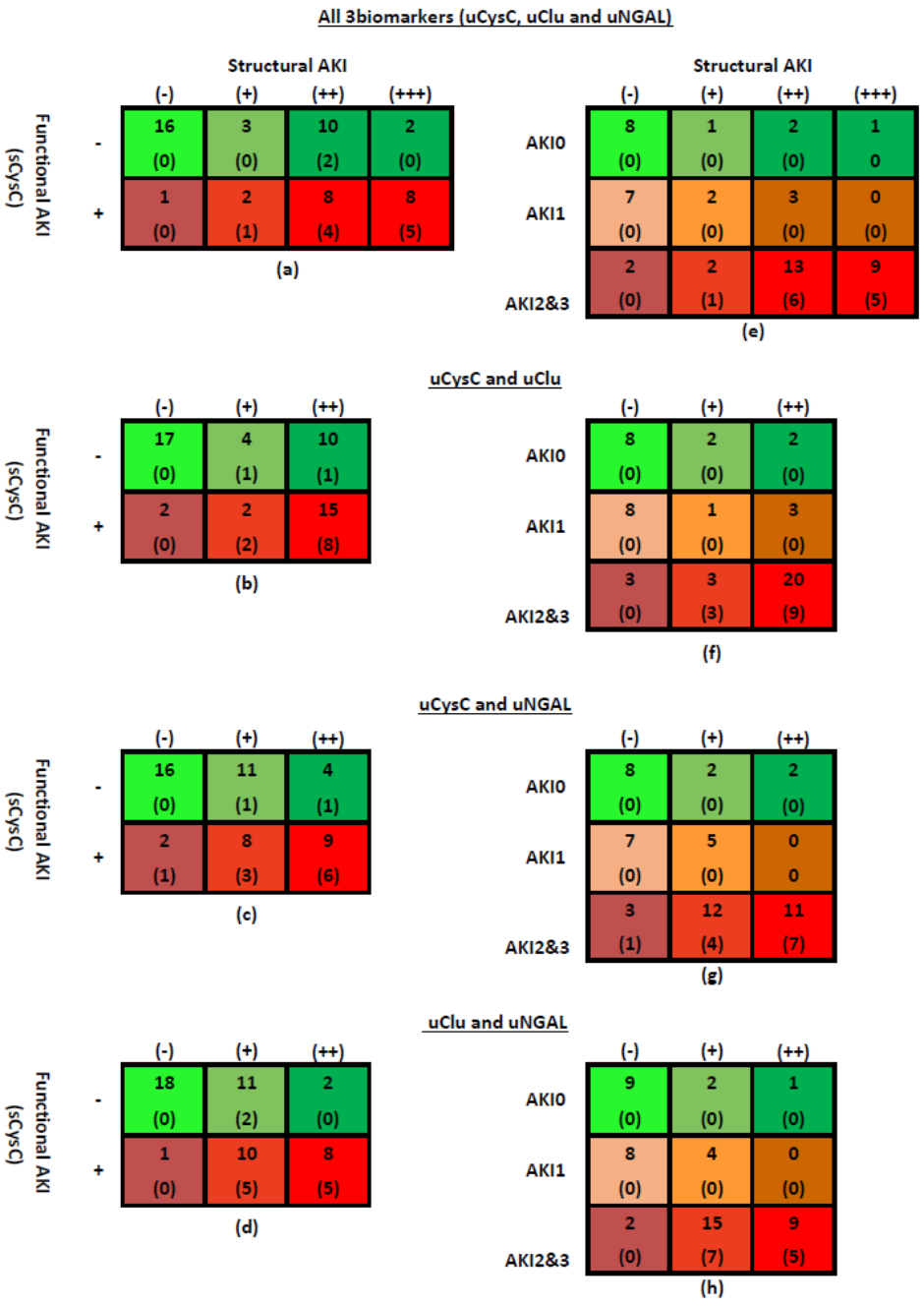



**Fig. 5 AKI classification according to combination of two or more structural biomarkers**

*More patients developed structural AKI when uCysC and uClu concentrations were above the cut-off values used for defining structural AKI. Structural AKI is defined as biomarker concentrations > 95th centiles values obtained from the healthy volunteers (uCysC: 70 ng/mg Cr; uClu: 420 ng/mg Cr; and uNGAL: 120 ng/mg Cr). Functional AKI in matrix (a-d) is defined as ≥ 50% increase in sCysC and in matrix (e-h) is based on AKIN definition. Each cell displays number of patients with number of deaths inside parenthesis. (-): structural biomarkers negative, (+): one structural biomarker positive. (++): two structural biomarkers positive, (+++): three structural biomarkers positive.*

**Table 3 Sensitivity and specificity for functional AKI or death based on structural biomarker thresholds**

| Biomarkers cut-off‡ and outcome | Sensitivity | Specificity | Positive likelihood ratio | Negative likelihood ratio | Diagnostic odds ratio |
|---|---|---|---|---|---|
| uCysC (>70 ng/mg Cr) | | | | | |
| Functional-AKI | 0.85 (0.66-0.94) | 0.65 (0.45-0.81) | 2.43 (1.36-4.36) | 0.24 (0.09-0.61) | 10.31 (2.63-40.5) |
| Death | 0.92 (0.65-0.98) | 0.49 (0.33-0.64) | 1.79 (1.25-2.55) | 0.17 (0.02-1.15) | 10.42 (1.22-89.13) |
| uClu (>420 ng/mg Cr) | | | | | |
| Functional-AKI | 0.81 (0.62-0.91) | 0.79 (0.60-0.91) | 3.88 (0.60-0.91) | 0.24 (0.11-0.55) | 15.96 (3.99-63.84) |
| Death | 0.83 (0.55-0.95) | 0.58 (0.42-0.72) | 1.98 (1.26-3.10) | 0.29 (0.08-1.05) | 6.88 (1.32-35.77) |
| uNGAL (>120 ng/mg Cr) | | | | | |
| Functional-AKI | 0.46 (0.29-0.65) | 0.87 (0.68-0.95) | 3.54 (1.14-11.0) | 0.62 (0.42-0.91) | 5.71 (1.36-20.06) |
| Death | 0.58 (0.32-0.81) | 0.78 (0.63-0.0.89) | 2.7 (1.24-5.87) | 0.53 (0.27-1.06) | 5.08 (1.26-20.36) |

*Functional AKI: AKIN stage 2/3 (≥100 increase in sCr), ‡ cut-offs are 95$^{th}$ centile values obtained from healthy volunteer data.*

### 3.7 Correlation of functional and structural biomarkers and among injury biomarkers

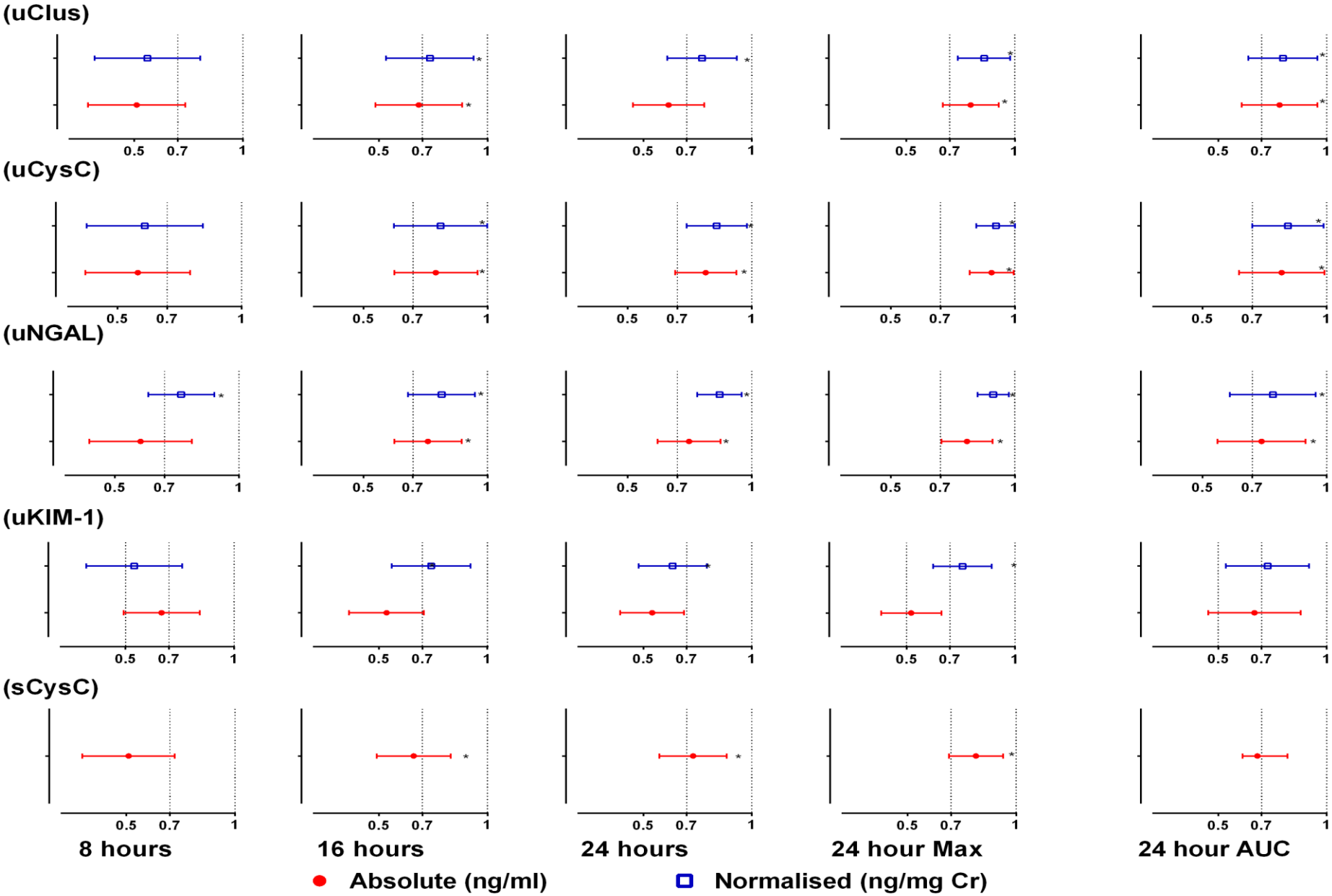
A positive correlation (r ≥0.5) was observed between sCr and the structural biomarkers, uCysC, uClu and uNGAL, 16 hours post ingestion. The correlation with sCysC was notably poor. As expected, the apparent 24 hour creatinine clearance correlated negatively with all of these injury biomarkers at 16 hours (Supplementary Fig. 6). The urinary injury biomarkers uCysC, uClu and uNGAL were correlated with each other at 16 hours and these correlations were improved by normalisation to urinary creatinine (Supplementary Fig. 6 and Supplementary Table 1).

### 3.8 Biomarker performance to diagnose functional AKI (AKIN 2/3) compared to healthy controls or NoAKI patients.

The diagnostic utility of biomarkers in detecting functional AKI (AKIN 2/3) was assessed using ROC analysis [against biomarker concentrations obtained from the healthy controls (Fig. 6, Supplementary Table 2) or against no-AKI patients (Supplementary Table 2). At 16-24 hours sCysC, uCysC, uClu and uNGAL revealed a moderate diagnostic performance to diagnose functional AKI. Both absolute and normalised concentrations of these four biomarkers at 16-24 hours had AUC-ROC values ≥0.7 (Fig. 6). The 24hrMax (peak) and 24hrAUC concentrations also showed similar diagnostic performance (Fig. 6). Sensitivity, specificity and cut-off values for each of these biomarkers at 16 and 24 hours are shown in Supplementary Table 2.

**Fig. 6 Biomarker performance to diagnose AKI after paraquat poisoning**

*AUC (±95% CI) as absolute (red) and normalised (blue) concentrations are shown for detection of AKI at different time points. The diagnostic performance in detection of functional AKI (AKIN stage ≥2) versus healthy controls is shown for 8, 16, 24 hours and maximum concentration within 24 hours, for each biomarker.*


(uClus)
(uCysC)
(uNGAL)
(uKIM-1)
(sCysC)
0.5
0.7
1
8 hours
16 hours
24 hours
24 hour Max
24 hour AUC
Absolute (ng/ml)
Normalised (ng/mg Cr)

318

319

The remaining biomarkers performed poorly in predicting functional AKI early (Supplementary Fig. 7). None of the urinary biomarkers distinguished AKIN stage 1 from no-AKI patients after paraquat (AUC≤0.5).

### 3.9 Sensitivity analysis

The sCr rise in paraquat poisoning may not be solely GFR related (Mohamed et al., 2015). As biomarker performances in this study were assessed using the AKIN consensus definition, a sensitivity analysis was conducted to assess the biomarker performance using an alternative sCysC definition (≥50% increase in sCysC). However, biomarker performance in detecting AKI based on either definition was similar (Table 4).

***Table** Error! No text of specified style in document. **Diagnostic performance of structural biomarkers (maximum concentration within first 24 hours) to diagnose functional AKI defined by sCr vs sCysC***

| Urinary biomarkers | AUC-ROC ((± 95% CI) | |
|---|---|---|
| | AKIN sCr definition for functional-AKI† | CysC definition for functional-AKI‡ |
| uClu | 0.80 (0.69-0.96) | 0.83 (0.66-0.92) |
| uCysC | 0.90 (0.80-1.00) | 0.91 (0.81-0.99) |
| uNGAL | 0.84 (0.74-0.95) | 0.84 (0.74-0.93) |
| uB2M | 0.82 (0.67-0.96) | 0.82 (0.70-0.82) |
| uKIM-1 | 0.53 (0.38-0.68) | 0.53 (0.39-0.67) |
| uIL18 | 0.54 (0.37-.76) | 0.56 (0.38-0.78) |
| uTFF3 | 0.75 (0.64-0.92) | 0.78 (0.62-0.89) |
| uOstP | 0.54 (0.44-0.74) | 0.60 (0.40-0.68) |
| sCysC | 0.81 (0.89-1.00) | 0.94 (0.69-0.94) |

*Diagnostic performance of each biomarker was assessed between biomarker concentrations in functional-AKI group (patients who developed functional AKI by either definition) versus healthy controls. † represents increase in serum creatinine (sCr) ≥ 100% (i.e. AKIN definition). ‡ represent increase in serum cystatin C (sCysC) ≥50% (CysC based AKI definition)*

## 4. Discussion

This is the first comprehensive multi-centre clinical study to evaluate the diagnostic performance of all seven FDA and EMEA "qualified" AKI biomarkers as well as uNGAL, uOstP and uIL-18 to diagnose nephrotoxicity after paraquat poisoning. Uniquely, biomarker diagnostic performance was assessed against two markers of renal function sCr and sCysC and also independently using thresholds derived from healthy individuals from the same population groups. Only the biomarkers urinary biomarkers uCysC, uClu, and uNGAL revealed modest diagnostic performance

for early detection of moderate to severe AKI as defined by ≥50% increase in sCysC, although uNGAL was increased for only 24 hours. However, added clinical utility of these biomarkers to sCr is limited in moderate to severe paraquat poisoning where most patients died within 24-48 hours of toxicity and sCr predicted death independent of nephrotoxicity in this group (AUC-ROC=0.9). Since serum creatinine is readily available and a cost-effective test in less developed countries where paraquat poisoning is common, it will remain a prognostic marker of poor outcome.

Poor biomarker performance in various clinical settings may be due to an imperfect gold standard used for defining AKI (Siew et al., 2011; Waikar et al., 2012). While histopathological change has been used as the gold standard for AKI diagnosis in toxicity studies, biomarker performance in clinical studies has usually been assessed against loss of renal function, as demonstrated by an increase in sCr or sCysC. In this study, we focussed on functional AKI as defined by a relative increase in sCr ≥100% sCr as it is widely used definition for assessing the biomarker performance (Waikar et al., 2012) or based on ≥ 50% sCysC (Nejat et al., 2010). Of patients meeting sCr definition of AKI (n=26), approximately 2/3 (n=19) showed a ≥50% rise in sCysC. Of 31 patients with <50% increase in sCysC, 19 were classified as AKI based on sCr definition over-estimating AKI mainly due to increase in sCr by non-renal mechanisms (Mohamed et al., 2015). Defining AKI based on relative increase in sCysC may be more appropriate in paraquat poisoning (Fahim et al., 2013) and serum CysC is less affected by non-renal factors (Bagshaw and Bellomo, 2010). Nevertheless, using ≥ 50% increase in sCysC to assess the biomarker performance also resulted in similar moderate diagnostic utility to AKIN definition in sensitivity analysis (Table 4). Validation of either sCr or sCysC as an appropriate surrogate marker of renal function awaits near real-time measurements of GFR.

We also examined the use of the recently-proposed matrix definition of AKI, which incorporates both structural and functional biomarkers (Murray et al., 2014). This requires identification of appropriate biomarker cutoffs (Pickering and Endre, 2013b). Since such cutoffs are contextual and therefore unavailable in this first major study of AKI biomarkers in paraquat poisoning, we used structural biomarker cutoffs based on the 95$^{th}$ centile value in healthy volunteers to define AKI (Basu et al., 2014). While there were differences amongst the best performing urinary biomarkers (uCysC, uClu, uNGAL), overall this resulted in more patients being diagnosed as having structural (65%) than functional AKI (Figs. 4 & 5, Table 2). However, only subjects with functional AKI died (Fig. 5). In the context of paraquat poisoning, renal damage alone (i.e., structural injury without significant loss of function) was not associated with death in any subjects. However, patient in this group had similar or higher serum paraquat level compare to functional AKI alone (i.e., functional AKI without structural injury). Nevertheless, the presence of

functional AKI based on sCr or sCysC definition marked both more severe renal damage and also more severe systemic events leading to a fatal outcome. This contrasts with the performance of urinary KIM-1 and NGAL in other AKI settings, such as septic or haemorrhagic AKI, where biomarker positive, creatinine negative subjects are at comparable risk of mortality and dialysis to creatinine-positive biomarker negative subjects (Haase et al., 2011; Nickolas et al., 2012). Presumably these differences in predicting risk of serious outcomes reflect the role of the individual biomarkers in the specific pathways of injury involved.

Since most renal injury biomarkers increased modestly and serum creatinine increased rapidly within 24 hours following moderate to severe paraquat poisoning, use of novel injury biomarkers to diagnose paraquat induced nephrotoxicity within first 2 days may be limited in clinical practice. Most patients in this group died within first 2 days of admission and sCr predicted mortality independent of loss of GFR. Therefore, novel biomarkers may not offer added diagnostic value to sCr. Early death following paraquat toxicity is mainly due to multi-organ failure with circulatory collapse (Dinis-Oliveira et al., 2008; Gawarammana and Buckley, 2011). Thus, initiating dialysis or renal replacement therapy based on sCr level in this group of patients may not be appropriate especially in poor resource settings. Furthermore, use of haemodialysis or haemofiltration to enhance paraquat elimination is not supported by clinical evidence in moderate to severe paraquat poisoning (Eddleston et al., 2003; Koo et al., 2002; Suzuki et al., 1993).

Among dozens of renal biomarkers studied (including FDA and EMEA “qualified” biomarkers) only a few biomarkers predicted AKI, which suggests the biomarker are specific to an injury pathway. Notably, uCysC, uClu and uNGAL were the most useful biomarkers in this study although their clinical significance in addition to sCr may be limited. Clusterin is expressed by dedifferentiated tubular epithelial cells as a cytoprotective agent following AKI (Rosenberg and Silkensen, 1995; Schwochau et al., 1998; Silkensen et al., 1997). Clusterin has a protective role against reactive oxygen species and its levels may increase in response to oxidative stress (Nath et al., 1994; Schwochau et al., 1998). Clusterin increases are therefore indirect evidence supporting oxidative stress as major mechanism of paraquat-mediated renal tubular toxicity (Nath et al., 1994; Rosenberg and Silkensen, 1995; Schwochau et al., 1998). In contrast, increases in uNGAL are indicative of a renal adaptation response to kidney injury (Mori et al., 2005). Upregulated NGAL may play a key role in preventing epithelial injury by scavenging free catalytic iron, the latter generating oxidative stress, by an anti-apoptotic effect, or by preserving N-cahedrin expression. NGAL also appear to upregulate heme oxygenase 1 which has anti-oxidant properties (Bolignano et al.,

2008; Mori et al., 2005). Increased levels of cystatin C (Bagshaw and Bellomo, 2010) following exposure to variety of nephrotoxins suggest that it serve as biomarkers of tubular injury and repairs.

In a rodent paraquat model of AKI defined by histopathological changes, uKIM-1 and uCysC were the best predictive biomarkers within 16-24 hours, and uClu performed poorly (Table 5) (Wunnapuk et al., 2013). The results for uClu and uKIM-1 were reversed in our clinical subjects. Treatment is generally ineffective and unlikely to explain the difference from animal models since death is rapid and due to fulminant multi-organ failure within hours to days, and the creatinine rise may be due to predominantly non-renal effects. The dissimilarities may also be due to difference in molecular forms of biomarkers between species (for example NGAL). NGAL in biological fluid is usually appears as monomeric and dimeric forms (Cai et al., 2010). NGAL monomeric form appears to be significantly correlated with tubular injury (Cai et al., 2010). However, most NGAL assays measure both forms. Sample handling and assay methods may also influence the results. The discrepancy may also be due to difference in defining AKI where animal study has used histopathology grading for defining AKI while human studies including this study uses sCr based AKI definition.

**Table 5 Comparative biomarker performance in predicting AKI within 24 hours in pre-clinical and clinical paraquat toxicity (Wunnapuk et al., 2013)**

| | **24 hour AUC-ROC (± 95% CI)** | |
|---|---|---|
| **Urinary biomarkers** | **Pre-clinical model (Wunnapuk et al., 2013)** | **Clinical setting** |
| uKIM-1 | 0.98 (0.93-1.0) | 0.63 (0.43-0.83) |
| uCysC | 0.88 (0.70-1.0) | 0.85 (0.72-0.98) |
| uNGAL | 0.85 (0.72-0.98) | 0.85 (0.72-0.98) |
| uB2M | 0.76 (0.54-0.98) | 0.67 (0.47-0.88) |
| sCysC | 0.60 (0.40-0.80) | 0.77 (0.61-0.93) |
| uClu | 0.54 (0.30-0.80) | 0.70 (0.51-0.88) |
| uIL18 | - | 0.68 (0.351-0.81) |

Subjects who survived beyond 3 days particularly following mild poisoning, urinary biomarkers generally predicted development of AKI, despite the limitations of the creatinine-based definitions already noted. Since absolute and normalised urinary CysC, clusterin and NGAL detect nephrotoxicity earlier than creatinine in this group (structural injury biomarker positive but functional biomarker negative) this may facilitate reno-protective intervention. The good biomarker performance in both clinical and pre-clinical studies (Table 5) adds further support for the utility of uCysC and clusterin (Wunnapuk et al., 2013). Furthermore, patient who survived beyond three days following paraquat poisoning develop pulmonary fibrosis (Gawarammana and Buckley, 2011) and this group may benefit from dialysis if started early. Clearly, further studies with long-term follow-up assessment are warranted to explore the utility of structural injury biomarkers in mild paraquat and its long term effects.

### 4.1 Strengths and limitations

Although this study has some notable strengths including being multicentre and of a larger size than previous paraquat nephrotoxicity studies and the exploration of a broad range of biomarkers at multiple time points, it also has limitations. These include the absence of direct measurement of GFR and the absence of absolute baseline functional or damage biomarker levels. Since a sensitivity analysis performed using some of the subjects included in this study showed that different baseline estimates did not result in under or over reporting of AKI incidence in paraquat poisoned patients (Fahim et al., 2013) and baseline sCysC was similar, this limitation is of less concern.

## 5. Conclusions

Serum cystatin C, urinary cystatin C, clusterin, and to a lesser extent NGAL showed modest diagnostic performance in moderate to severe paraquat poisoning. Since most patients died in this group and sCr predicted mortality independent of nephrotoxicity, clinical utility of renal injury biomarkers in addition to sCr is limited. Serum cystatin C should be used to diagnose AKI in paraquat poisoning. Increase in specific injury biomarkers identified mechanistic pathways of nephrotoxicity in proximal tubules. Use of structural injury biomarkers within 16-24 hours of ingestion may guide early intervention for reno-protection in mild paraquat poisoning. Point-of-care biomarker detection should accelerate early intervention in these groups of patients in rural Asia.

## Conflict of interest statement

We declare there is no conflict of interest.

**Acknowledgments**

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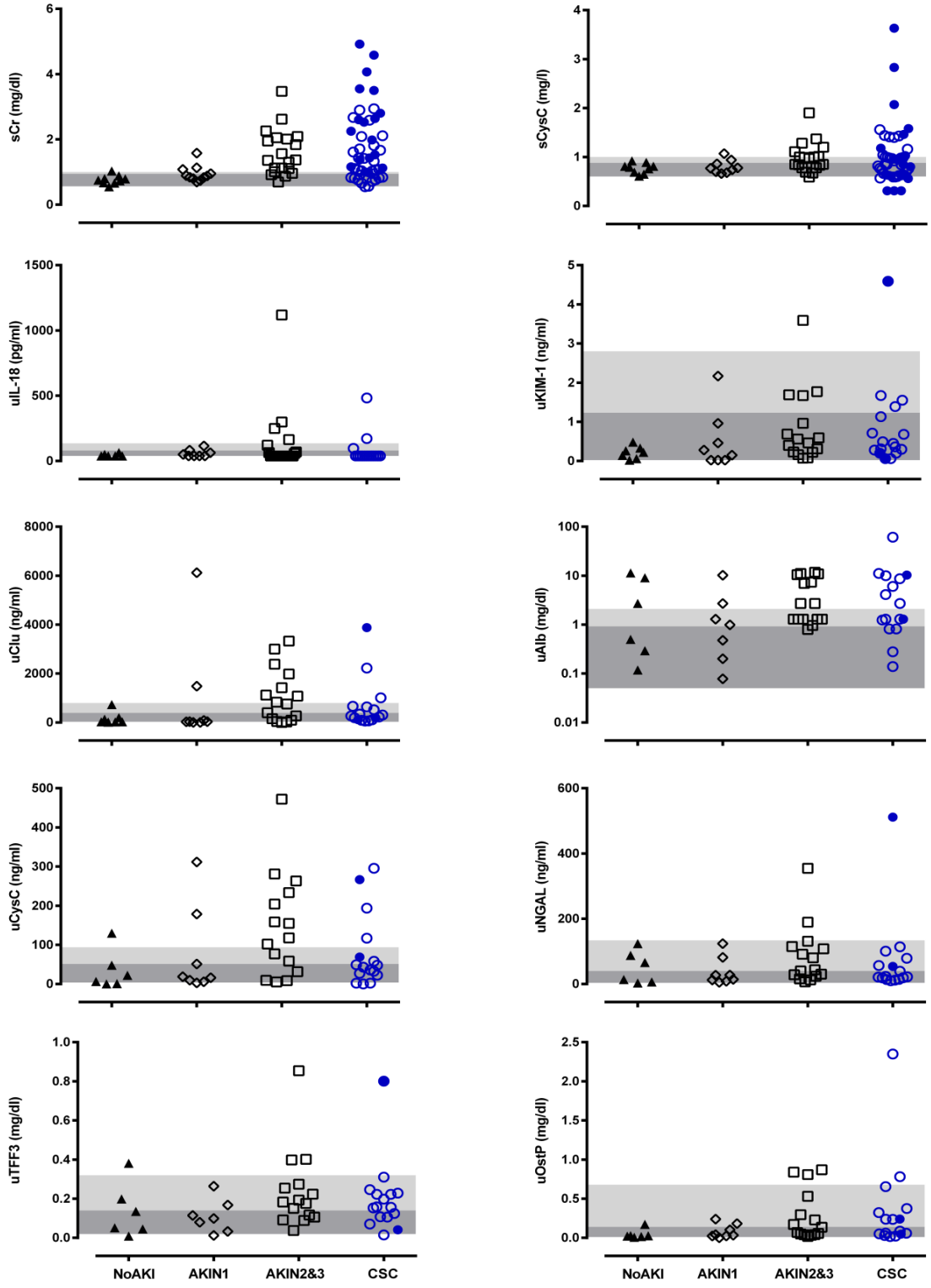
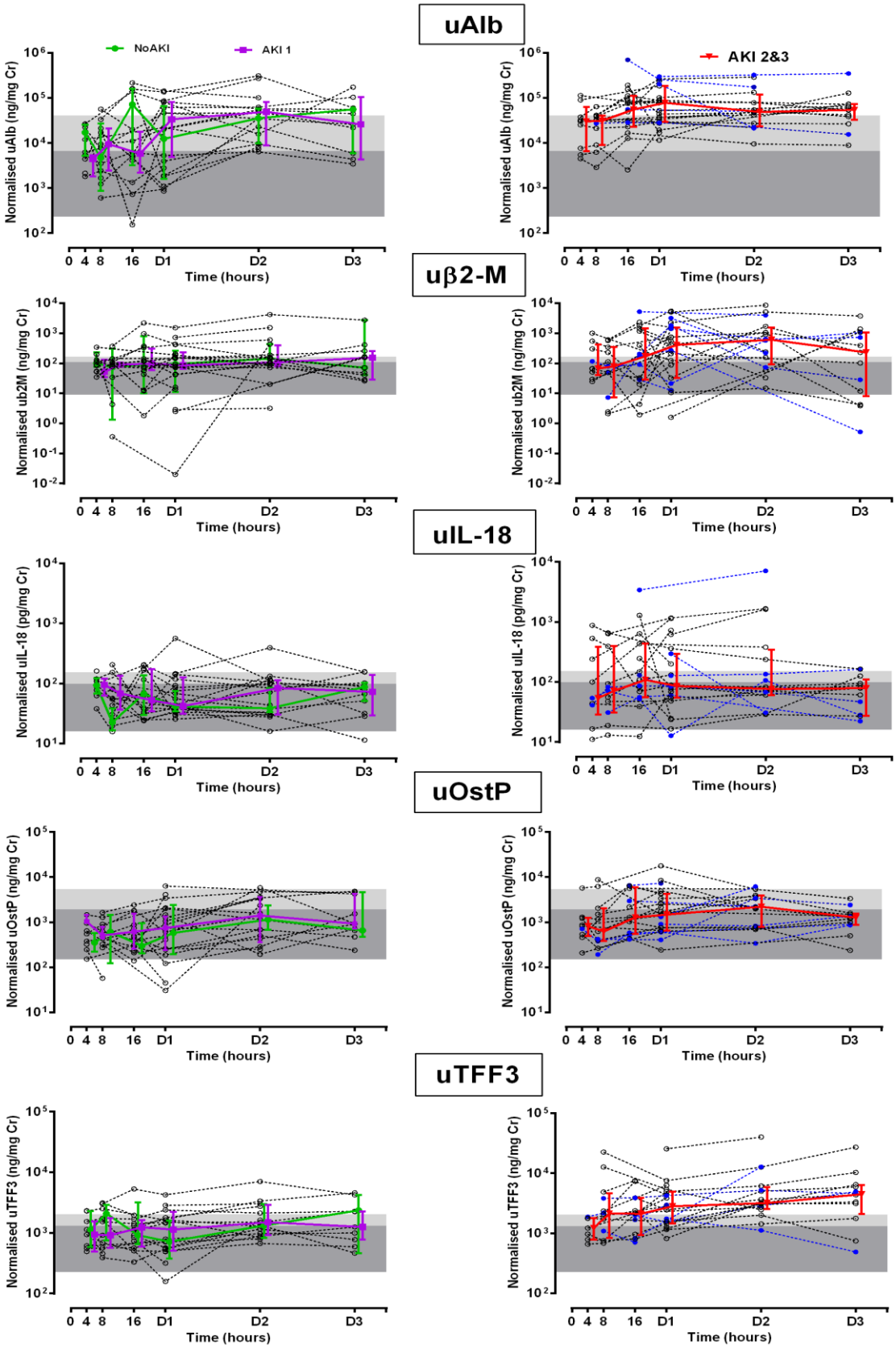
## Appendix A. Supplementary data

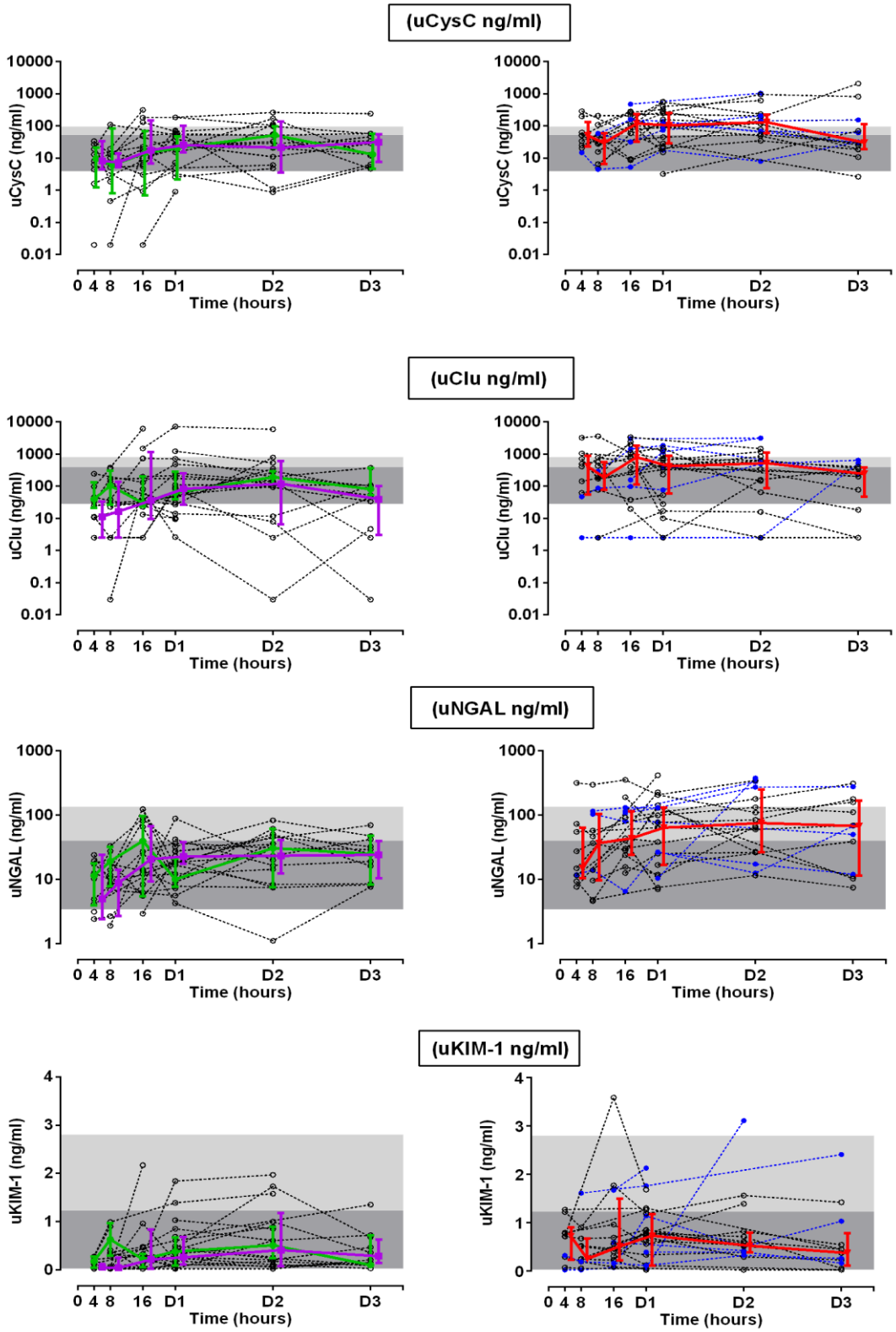



**Supplementary Fig. 1. Scatter plots of urinary biomarkers at 16 hours from the main cohort according to AKIN staging and highest biomarker concentrations in patients from cross sectional cohort (CSC).**
*Thirty-one patients were excluded from the main cohort as they provided only one blood or urine sample; some only provided either a blood or urine sample. These graphs show 24-hour maximum biomarker concentration profiles in CSC were similar to that of patients in the main cohort. The solid blue symbol depicts biomarker concentrations in patients who died.*

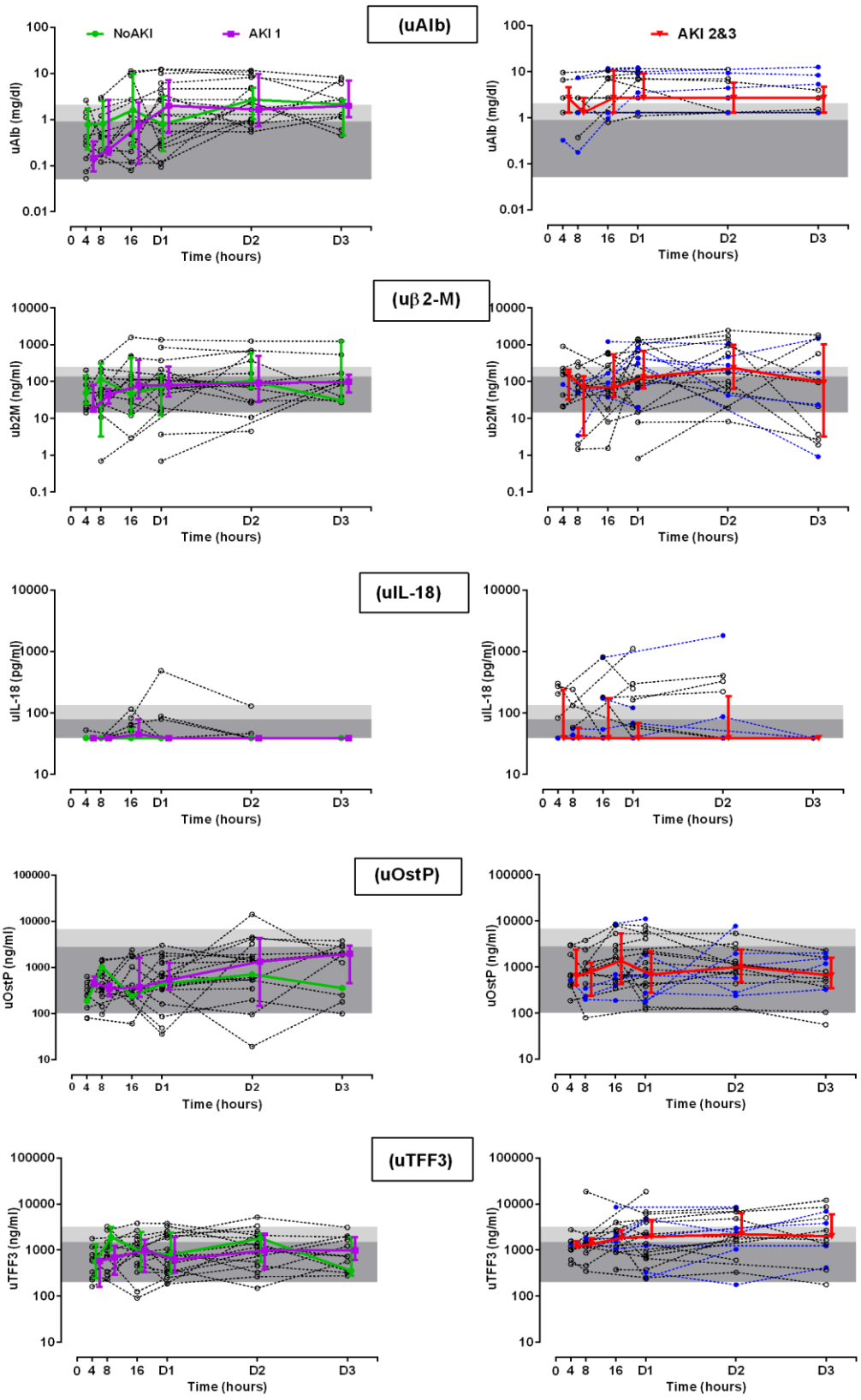



***Supplementary Fig. 2. Serial biomarker profiles (normalised to urinary creatinine) following paraquat poisoning, relative to development of AKI***

*Normalised biomarker concentrations of uAlb, uβ2M, uIL-18, uOstP and uTFF3 are plotted for each patient (dashed line). Blue dashed lines depict patients who developed AKI stage 2 and other patients in this group represent AKI stage 3. The dark bolded lines represent median (±IQR) changes in each group (black No-AKI group; green AKI stage 1 group, red AKI >=2 group). The shaded area illustrates the normal range based on respective biomarker concentration measurements in healthy individuals (dark grey: 5th to 75th centiles; light grey: 75th to 95th centiles)*

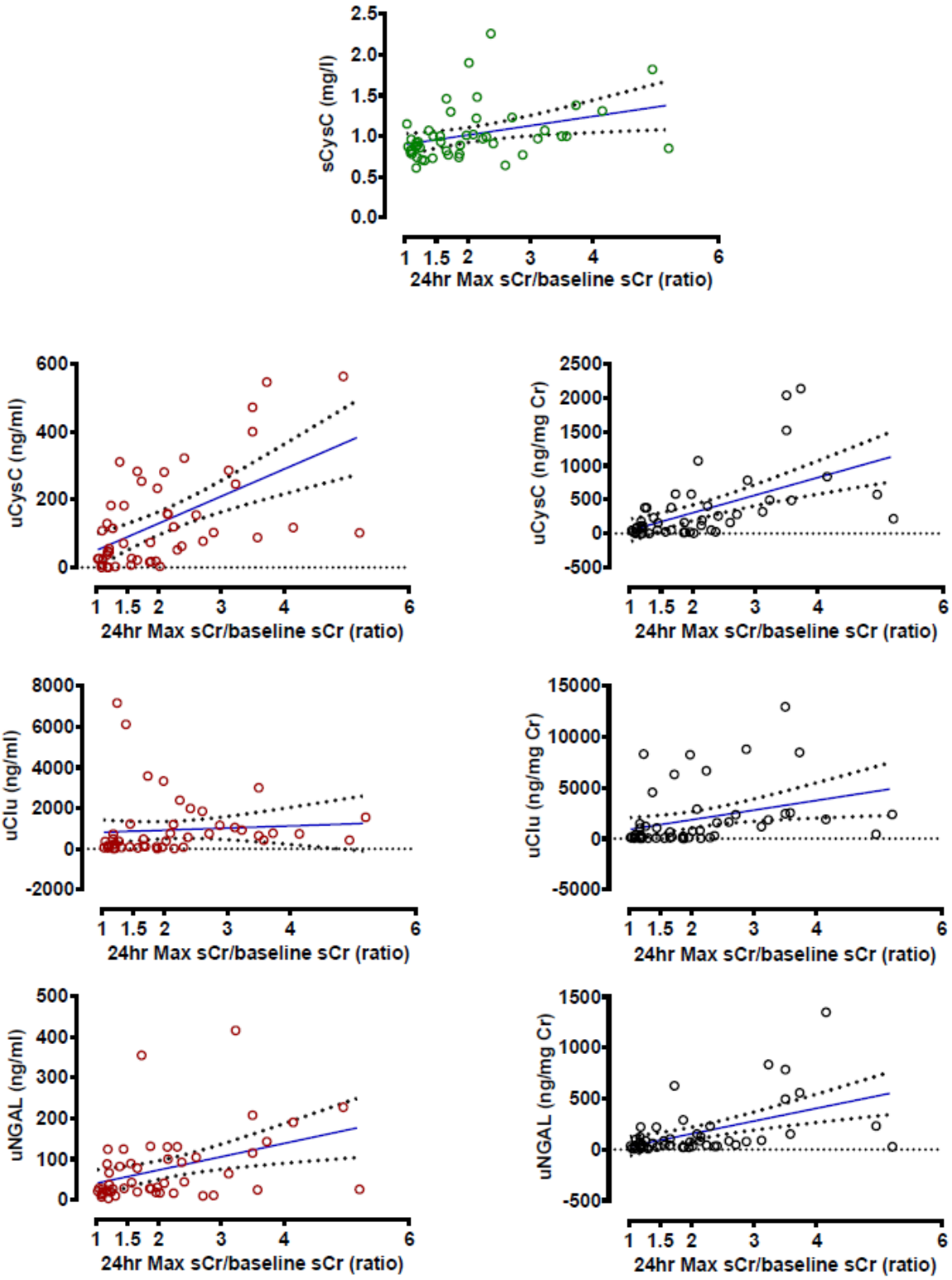



**Supplementary Fig. 3. Absolute biomarker concentration profiles following paraquat poisoning.** *Individual patient's absolute concentrations of uCysC, uClu, uKIM-1 and uNGAL are shown (dashed lines). Blue dashed lines depict patients who developed AKI stage 2 and other patients in this group represent AKI stage 3. The dark bolded line in each graph represents the median (± IQR) change in each group (green line-No-AKI group; purple line-AKI stage 1 group, red line-AKI≥2 group). The grey shaded area illustrates the normal range based on respective biomarkers measured in healthy individuals (dark grey area-5th to 75th centiles; light grey area-75th to 95th centiles of the normal range).*




**Supplementary Fig. 4. Absolute serial biomarker profiles following paraquat poisoning, relative to development of AKI.**

*Absolute biomarker concentrations of uAlb, uβ2M, uIL-18, uOstP and uTFF3 are plotted for each patient (dashed line). Blue dashed lines depict patients who developed AKI stage 2 and other patients in this group represent AKI stage 3. The dark bolded lines represent median (±IQR) changes in each group (black No-AKI group; green AKI stage 1 group, red AKI >=2 group). The shaded area illustrates the normal range based on respective biomarker concentration measurements in healthy individuals (dark grey: 5th to 75th centiles; light grey: 75th to 95th centiles)*



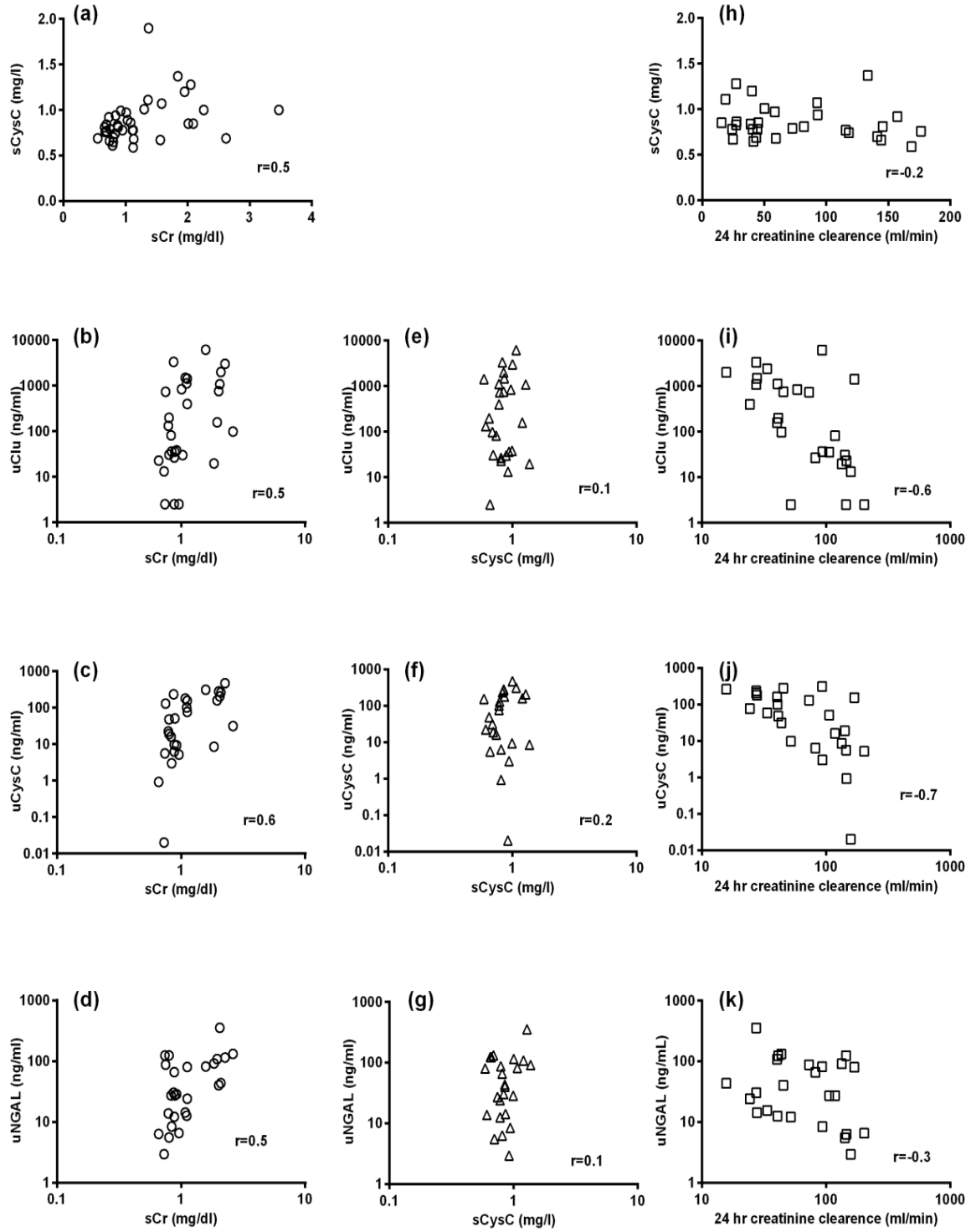

**Supplementary Fig. 5. Peak urine and serum biomarker concentration versus maximum change in sCr**

*The maximum 24 hour absolute sCysC and both absolute and normalised concentrations of uCysC, uClu and uNGAL are shown. The scatter plot displays maximum 24 hour peak concentrations of biomarker against 24 hour peak sCr to baseline sCr ratio. The dotted lines show 95% CI of the slope. The slope of each relationship illustrates the enhancement associated with normalising each biomarker as a function of relative change in serum creatinine.*

**Supplementary Table 1. Correlations (Spearman) among injury biomarkers after paraquat poisoning**

| | Absolute biomarker concentrations | | Normalised biomarker concentrations | |
|---|---|---|---|---|
| | **uClu** | **uCysC** | **uClu** | **uCysC** |
| **16 hour biomarker concentrations** | | | | |
| **uCysC** | 0.89* | | 0.90* | |
| **uNGAL** | 0.31 | 0.46* | 0.54* | 0.70* |
| **24hrMax concentrations†** | | | | |
| **uCysC** | 0.76* | | 0.83* | |
| **uNGAL** | 0.23 | 0.58* | 0.44* | 0.73* |
| **24hrAUC‡** | | | | |
| **uCysC** | 0.83* | | 0.89* | |
| **uNGAL** | 0.37* | 0.61* | 0.46* | 0.66* |

*†Peak biomarker concentration within 24 hours of ingestion. ‡ Total area under the concentration curve at 24 hours.*
** $p < 0.05$ (significant correlation)*



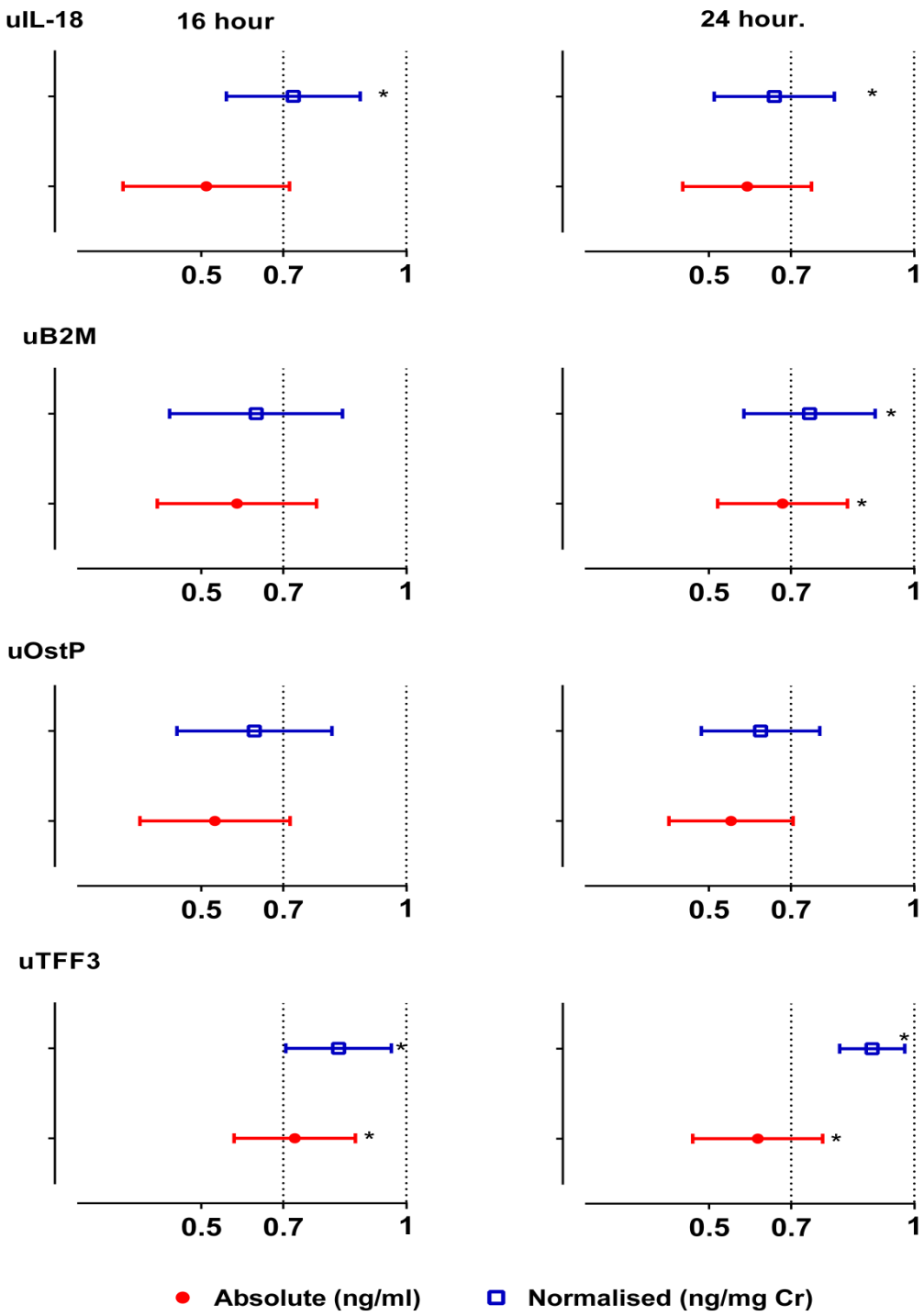

**Supplementary Fig. 6. Correlations amongst injury and functional biomarkers at 16 hours**

*The correlation (Spearman) plots depict injury biomarkers (uClu, uCysC and uNGAL) vs sCr (Fig. a to d), sCysC (Fig. e to g) and 24 hour apparent creatinine clearance (Fig. h to k). Positive correlations were obtained for injury biomarkers compared to sCr; negative correlations were seen with 24 hour creatinine clearance. Serum CysC was weakly correlated with injury biomarkers.*

**Supplementary Table 2: Diagnostic characteristics of biomarkers in early diagnosis of functional-AKI (stage 2/3) in paraquat poisoning.**

| **AKI≥2 versus healthy individuals** | | | | | | **AKI≥2 versus all non-AKI patient controls** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Biomarkers** | **AUC-ROC (95% CI)** | **p** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Cut-off** | **AUC-ROC (95% CI)** | **P** | **Sensitivity (95% CI)** | **Specificity (95% CI)** | **Cut-off** |
| **16 hours post ingestion (absolute concentration)** | | | | | | **16 hours post ingestion (absolute concentration)** | | | | |
| sCysC (mg/l) | 0.66 (0.49-0.83) | <0.05 | 68 (43-87) | 52 (37-66) | >0.84 | 0.76 (0.59-0.94) | <0.05 | 74 (48-91) | 78 (40-97) | > 0.82 |
| uClu (ng/ml) | 0.69 (0.49-0.88) | <0.05 | 69 (41-89) | 60 (43-74) | >266 | 0.79 (0.61-0.98) | <0.05 | 75 (48-93) | 71 (29-96) | >144 |
| uCysC (ng/ml) | 0.79 (0.62-0.96) | <0.001 | 73 (44-92) | 73 (57-86) | >46 | 0.82 (0.63 -1.00) | <0.05 | 73 (45-92) | 83 (36-100) | > 53 |
| uNGAL (ng/ml) | 0.79 (0.66-0.9) | <0.001 | 80 (52-74) | 74 (59-86) | >23 | 0.66 (0.37-0.94) | >0.05 | 67 (38-88) | 50 (12-88) | > 29 |
| **24 hours post ingestion (absolute concentration)** | | | | | | **24 hours post ingestion (absolute concentration)** | | | | |
| sCysC (mg/l) | 0.73 (0.58-0.88) | <0.01 | 68 (45-86) | 72 (58-84) | >0.88 | 0.77 (0.61-0.93) | <0.05 | 68 (45-86) | 70 (35-93) | > 0.86 |
| uClu (ng/ml) | 0.61 (0.45-0.78) | >0.05 | 67 (45-84) | 67 (50-80) | >280 | 0.70 (0.51-0.88) | >0.05 | 70 (49-87) | 70 (35-93) | > 194 |
| uCysC (ng/ml) | 0.81 (0.68-0.93) | <0.0001 | 70 (47-87) | 70 (54-84) | >45 | 0.85 (0.72-0.98) | <0.01 | 74 (52-90) | 70 (35-93) | > 41 |
| uNGAL (ng/ml) | 0.78 (0.65-0.90) | <0.001 | 74 (52-90) | 74 (58-86) | >24 | 0.85 (0.72-0.98) | <0.01 | 78 (56-92) | 70 (35-93) | > 15 |

*Comparisons were made between stage 2/3 (AKI ≥2) vs healthy controls and AKI ≥2 vs no-AKI patient controls. Data are presented only for the best performing biomarkers to diagnose AKI.*

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520

521 **Supplementary Fig. 7. Biomarker performances to diagnose AKI following paraquat poisoning**

522 *AUC (±95% CI) as absolute (red) and normalised (blue) concentrations are shown for detection of AKI at 16*

523 *and 24 hours. Diagnostic performance was assessed between patients in functional AKI (AKIN stage ≥2) group*

524 *and healthy controls at each time point (* $p<0.05$).*

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