Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers

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Abstract

Accidental or intentional ingestion of glyphosate surfactant-based herbicides, like Roundup®, leads to nephrotoxicity as well as death. In this study, a panel of kidney injury biomarkers was evaluated in terms of suitability to detect acute kidney injury and dysfunction. The Roundup® intoxication model involved oral administration of glyphosate to rats at dose levels of 250, 500, 1200 and 2500 mg/kg. Urinary and plasma biomarker patterns were investigated at 8, 24 and 48 hours after dosing. Biomarkers were quantified by absolute concentration; by normalising to urine creatinine; and by calculating the excretion rate. The diagnostic performances of each method in predicting of acute kidney injury were compared. By Receiver Operating Characteristic (ROC) analysis of the selected biomarkers, only urinary kidney injury molecule-1 (KIM-1) best predicted histological changes at 8 h (best cut-off point > 0.00029 µg/ml). Plasma creatinine performed better than other biomarkers at 24 h (best cut-off point > 0.21 mg/dl). Urinary KIM-1 was the best early biomarker of kidney injury in this glyphosate-induced nephrotoxicity model.

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine, CAS # 1017-83-6] is the active ingredient in glyphosate-surfactant herbicides (GPSHs) which are used extensively as non-selective herbicides (Bradberry et al., 2004; Lee et al., 2009). GPSHs usually consist of glyphosate salts (e.g. isopropylamine/IPA, potassium, and di-ammonium salt), surfactant, anti-foaming agents, colour agents, biocides and inorganic ions for pH adjustment (Bradberry et al., 2004; Lee et al., 2009). Commercial products contain approximately 1 to 40%, or sometimes more, glyphosate (Bradberry et al., 2004; Roberts et al., 2010). Human intoxication with this herbicide results not only from the active ingredient but also from its complex and variable mixture. Thus polyoxyethyleneamine (POEA) is one of the surfactants extensively used and it has a toxic effect in its own right (Bradberry et al., 2004; Roberts et al., 2010). Glyphosate is one of the most commonly used herbicides in the world, especially in South East Asia (Cobb and Reade, 2010). Even though GPSHs are classified by the US Environmental Protection Agency as being in the least toxic category (IV), ingestion of a sufficient amount of GPSHs can lead to death, with reported case-fatality ranging from 3 to 30% (Ahsan et al., 2008; Chen et al., 2009; Lee et al., 2000; Nagami et al., 2005; Roberts et al., 2010; Sawada et al., 1988; Talbot et al., 1991; Tominack et al., 1991). The clinical outcomes of GPSH ingestion vary depending on the formulation, but include multi-organ toxicity, with nephrotoxicity, hepatotoxicity, gastrointestinal, cardiovascular and respiratory effects (Lee et al., 2008; Lee et al., 2009; Roberts et al., 2010).

Patients who die from GPSH ingestion generally develop acute kidney injury (AKI). The kidney may also be an organ for excretion of GPSH components (Moon and Chun, 2010; Roberts et al., 2010; Sribanditmongkol et al., 2012). Thus, early evidence of kidney injury could be used to predict the risk of a fatal outcome in GPSH toxicity.

Plasma (pCr) or serum creatinine (sCr) and blood urea nitrogen (BUN) have usually been used as indicators, or biomarkers, for kidney injury in human GPSH intoxication (El-Shenawy, 2009; Lee et al., 2008; Moon and Chun, 2010; Talbot et al., 1991; Tominack et al., 1991). Recently, several studies have suggested that pCr is not sensitive enough to detect early kidney injury or predict subsequent functional change (Sherwin et al., 2012; Toto, 1995; Vaidya et al., 2008b; Woitas et al., 2000). Other biomarkers including interleukin-18 (IL-18), cystatin-C (Cys-C), kidney injury

molecule-1 (KIM-1), β_2-microglobulin (β_2-M), albumin (Alb), neutrophil gelatinase-associated lipocalin (NGAL) and osteopontin (Opn) have been proposed for the early and sensitive detection of AKI and functional loss (Coca and Parikh, 2008; Coca et al., 2008; Ferguson et al., 2008; Nguyen and Devarajan, 2008; Noiri et al., 2009; Vaidya et al., 2008a; Vaidya et al., 2008b), and may have greater sensitivity than pCr. Some of these biomarkers are specific to the degree and site of kidney injury, while some indicate kidney dysfunction (e.g. Cys-C), but each biomarker shows a different time-profile after intoxication (Bonventre et al., 2010; Hoffmann et al., 2010). To the best of our knowledge, there is no systemic study comparing the performance of these markers for predicting GPSHs induced AKI.

The current study aimed to find sensitive and specific biomarkers to diagnose the early phase of AKI and to localise the site of damage in rats after GPSH exposure. Fourteen candidate urinary biomarkers; uKIM-1, clusterin (uClu), uAlb, uβ_2-M, uCys-C, uNGAL, uOpn, vascular endothelial growth factor (uVEGF), tissue inhibitor of metalloproteinases (uTIMP-1), α-1-acid glycoprotein (uAPG), chemokine interferon-inducible protein 10 (uIP-10), epidermal growth factor (uEGF), Calbindin-D28 (Cal) and alpha glutathione S-transferase (uGST-α) were examined in this study. The plasma biomarkers Cys-C (pCys-C) and NGAL (pNGAL) were also investigated. Biomarker patterns at different time points after treatment of different doses of glyphosate were defined and correlated to kidney injury (defined by histological changes) and kidney function (defined by creatinine level). Biomarkers were quantified in three different ways: absolute concentration, by normalising to urine creatinine (uCr) and by calculating the excretion rate. The diagnostic performances of each method in predicting of AKI were compared.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g) were purchased from the Animal Resources Centre (Western Australia, Australia) and housed on a 12 h light/dark cycle. The animals were allowed free access to food (standard laboratory chow) and water. Rats were fasted by withdrawing food overnight (12 h) before the experiments.

2.2. Preparation of glyphosate solution

The commercial product of glyphosate (Concentrate Roundup® Weedkiller, Monsanto Australia Limited, Melbourne, Australia) containing 360 g/L of glyphosate as the isopropylamine salt was used in the study in a manner similar to previous published work (El-Shenawy, 2009; Lee et al., 2009; Talbot et al., 1991). It was diluted with water into 4 different concentrations (25, 50, 120 and 250 mg/ml) and was used for 4 different dose groups (250, 500, 1200 and 2500 mg/kg). The doses were chosen as being 5%, 10%, 25% and 50% of 7 day LD_{50} in rats (5000 mg/kg) (Williams et al., 2000).

2.3. Experimental Design

All experiments were approved by the University Animal Ethics Committee (Health Sciences) in the University of Queensland (AEC approval number: PAH/370/10/NHMRC). The rats were randomly divided into the 4 dose groups with 4 rats in each group. Glyphosate solution was administered orally. Animal experiments were performed according to the OECD425 up-down procedure guideline (OECD, 2008). Four rats were given only water and used as controls. After administration, rats were placed in individual metabolic cages and urine samples were collected on dry ice at intervals of 0-8 h, 8-24 h and 24-48 h. Blood was collected from the tail vein of each rat at 8 and 24 h. At 48 h, the rats were sacrificed and blood was collected from the vena cava. Blood samples were centrifuged at 3,000 rpm for 10 min at 4°C to collect plasma. Plasma and urine samples were stored at -70°C until biomarker analysis.

2.4. Kidney biomarker analysis in urine and plasma

The levels of Cr, Clu, Opn, KIM-1, β2-M, Cys-C, Alb, NGAL, Cal, GSTα, IP-10, TIMP-1, VEGF, AGP, EGF in urine and Cr, Cys-C NGAL in plasma were determined for control and glyphosate-treated animals.

Cr levels in plasma and urine were determined by the Jaffe method using a Beckman DxC800 general chemistry analyser (Beckman Coulter, Fullerton, CA, USA), which was performed in the Chemical Pathology Department at the Princess Alexandra Hospital (Brisbane, Queensland, Australia).

pNGAL and pCys C were quantified by ELISA [R and D Systems, Inc.] in batches following the manufacturer's instructions. Urinary Clu, Opn, KIM-1, β2-M, Cys-C, Alb, NGAL ,Cal, GST-α, IP-10, TIMP-1, VEGF, AGP and EGF were determined using Millipore Milliplex Rat Mag Kidney Toxicity Panels 1 and 2 on the Luminex®

xMAP® platform (EMD Millipore Corporation, Billerica, MA, USA), following the manufacturer's instructions. Intra- and inter-assay variability was lower than 10% and 15%, respectively. These assays were performed by the Kidney Biomarker Research Group at the Prince of Wales Hospital, Sydney, NSW, Australia.

2.5. Histological analysis

The left kidney was removed from each animal and fixed in 10% neutral buffered formalin for 24 h, routinely embedded in paraffin, sectioned, then stained with hematoxylin and eosin or the *in situ* enzymatic assay ApopTag (ApopTag® Peroxidase *In situ* Apoptosis Detection Kit, Merck-Millipore Corporation, Billerica, MA) for evaluation of necrosis or apoptosis, respectively. ImageScope image analysis/viewer software version 10.1 (Aperio, Vista, CA) was used to analyse the digitally-scanned slides. Slides were viewed blinded to treatments and results reviewed under the supervision of an experienced pathologist. For each rat, twelve histological fields at x200 magnification were randomly selected in each slide and histological changes were quantified. Means from ten fields were used after excluding the lowest and highest values as outliers. Characteristics used to identify apoptosis included cellular rounding and shrinkage, nuclear chromatin compaction especially along the nuclear envelope in a crescentic manner, membrane-bound cellular blebbing and formation of apoptotic bodies. Characteristics for necrosis were cells with swollen eosinophilic cytoplasm, disrupted cell and organelle membranes, and lytic or densely pyknotic nuclear changes (Gobe, 2009). Necrotic and apoptotic cell counts per microscopic field were converted to cells per square millimetre in order to obtain quantitative determination of the degree of toxicity. Cell death was normalised for background cell death and was presented as normalised cell death.

2.6. Data analysis

Receiver Operating Characteristic (ROC) analysis was employed to compare and define biomarker performances (i.e. sensitivity and specificity) and was calculated for absolute biomarker concentration, biomarker normalised to uCr and biomarker excretion rate data obtained from control vs. treated animals and from animals without vs. those with abnormal histological findings in the kidney. An area under the curve (AUC)-ROC value of 0.9-1.0 indicates excellent performance, 0.8-0.89 good performance, 0.7-0.79 fair performance, 0.6-0.69 poor performance and < 0.6 insignificant performance (Haase-Fielitz et al., 2009). The normalised biomarker concentrations were obtained by dividing the biomarker concentration (μg/ml) by the

uCr concentration (Cr levels were converted from mg/dl to µg/ml). Urinary biomarker excretion rates were calculated by: excretion rate (µg/h) = [concentration (µg/ml) ×volume of urine (ml)]/time interval (h).

The changes in Cr clearance, Cys C and NGAL apparent clearances were also determined as well as the correlation between Cr clearance and NGAL and Cys-C apparent clearances. Biomarker clearances were calculated by: biomarker clearance (ml/min/kg) = [urine flow rate (ml/min) × urine concentration of the biomarker (µg/ml)]/plasma concentration of the biomarker (µg/ml).

2.7. Statistics

As biomarker concentrations are not normally distributed, nonparametric analysis (Mann-Whitney rank-sum test) was used to compare histological changes and biomarker levels found in treatment groups with time-matched controls. Correlations between injury biomarkers and functional markers were determined using Spearman analysis with a 95% confidence interval. Significance was defined as $p < 0.05$. All statistical analyses were conducted using GraphPad Prism version 6.01 (GraphPad Software, San Diego, USA). Comparison of the AUCs was performed using the method described by Delong et al. (DeLong et al., 1988).

3. Results

3.1. Effect of Roundup® treatment on kidney histology

A significant increase in necrotic and apoptotic cells per mm^2 was observed (Fig. 1A and B). Necrotic and apoptotic cells were localised primarily in the tubular epithelium of the proximal straight tubule and thick ascending limb of the loop of Henle in the outer medulla than in nephron structures of the cortex. It should be noted that animals treated with 1200 and 2500 mg/kg of glyphosate showed severe dehydration and diarrhoea. A lower level of cell damage seen in these high dose groups (Fig. 1C) could be a result of low absorption of glyphosate and its surfactants via gut wall due to diarrhoea. In comparison with control kidney (Fig. 1D), Roundup® administration induced marked histological changes, including proximal and distal tubular necrosis and glomerular toxicity (Fig. 1E and F) and apoptosis (Fig. 1G). To our knowledge, this present study is the first to demonstrate apoptosis induced by Roundup® in an *in vivo* model of nephrotoxicity.

3.2. Plasma biomarker changes in the Roundup®-treated rats

As shown in Table 1 and Supplementary Fig.1, the level of all plasma biomarkers increased 1 day after Roundup® ingestion, especially at the dose of 2500 mg/kg. In this dose group, pCr levels increased (1.7-fold the time-matched controls) as early as 8 h after treatment. Moreover, pNGAL levels were increased 7-fold relative to controls after 8 h. Similarly, pCys-C levels increased between 1.9- and 2.4-fold after 8 h.

3.3. Urinary biomarker changes in the Roundup®-treated rats

Compared to control levels there was a significant increase in uKIM-1(3.2- and 4.4-fold at 48 h for 500 and 2500 mg/kg of glyphosate, respectively), and uVEGF levels (13- and 20-fold) at the 24- and 48-h time points with 2500 mg/kg of glyphosate ($p < 0.01$, compared to control) as shown in Table 1 and Supplementary Fig.2. In the 250 mg/kg Roundup®-treated rats group, no significant changes were observed for any biomarkers in both urine and plasma, although some histological changes were noted in this group of rats (Supplementary Fig. 3). For creatinine-normalised biomarker concentrations, several urinary biomarkers including uβ2-M, uCys-C, uAlb, uKIM-1, uClu, uNGAL and uAGP showed significant responses to Roundup® (Supplementary Fig. 4 and 5) after day 1 of exposure. The uCys-C:Cr and uAGP:Cr values elevated as early as 8 h after dosing (1.5- and 1.8-fold, respectively). Urinary β2-M:Cr, uAlb:Cr, uKIM-1:Cr and uNGAL:Cr values were elevated between 2.4 and 4-fold relative to the control values within day 1. The urinary excretion rates did not significantly amplify the signal or improve detection of AKI for any biomarkers (Supplementary Fig. 6 and 7).

3.4. Apparent biomarkers and creatinine clearances

The changes in Cr clearance, Cys-C and NGAL apparent clearances and their correlation with each other are shown in Fig. 2A and B. There was a significant correlation between Cr clearance and apparent clearances of NGAL and Cys-C (Spearman r = 0.67 $p < 0.0001$ and r = 0.50, $p = 0.004$ for NGAL and Cys-C, respectively). Plasma Cys-C levels directly correlated with pCr (Fig. 2C) (Spearman r = 0.48). However, pCys-C levels were not significantly related to Cr clearance (Fig. 2D). There was no relationship between other urinary biomarker levels and pCr or Cr clearance.

3.5. Kidney biomarker concentration-time profiles

The time profile of biomarker level changes after Roundup® ingestion is shown in Fig. 3. The biomarker levels at time 0 were obtained from the analysis of control rats. A normal range for each biomarker was defined as that biomarker's level in the control group. The increase in uKIM-1, uClu, uVEGF and pCys-C, pNGAL and pCr demonstrated some dose-dependence, most obviously at the highest dose. Urinary KIM-1 levels and pCr (Fig. 3A and F) were higher than the normal range as early as 8 h after Roundup® dosing, and their levels increased until 48 h after dosing. Similarly, the uClu and uVEGF levels rose soon after dosing and remained higher than the baseline. For these biomarkers, however, this was found only in 2500 mg/kg of glyphosate dose group (Fig. 3C and E). In contrast, pNGAL and pCys-C levels only started to increase as late as 24 or 48 h after treatment (Fig. 3B and D).

3.6. ROC analysis and biomarker performance

Table 2 lists the injury biomarkers with excellent and good performance, based on ROC analysis. The AUC-ROCs were slightly variable for a given biomarker when comparing absolute urinary concentration, normalised concentration and excretion rate (Fig. 4). Urinary KIM-1 yielded the best performance at 8 h (AUC-ROC of 0.85) for the prediction of histological injury in kidney. Plasma Cr outperformed other biomarkers (AUC-ROC of 0.89 and 0.96 at 24 and 48 h, respectively) for the prediction of kidney damage after 24 h (Supplementary Fig. 8). Excretion rate and normalised biomarker concentration did not improve biomarker performances in the diagnosis and prognosis of AKI.

4. Discussion

In this study, biomarker and histological changes in the kidney were examined in rats after sub-lethal doses of Roundup® to identify suitable biomarkers able to detect the early stages of kidney injury. The performances of biomarkers in the US-FDA approved kidney injury biomarker panel, proposed by the PSTC, were examined. This panel includes the conventional functional marker of kidney function (pCr) but also several biomarkers including uKIM-1 and uCys-C. There was no clear relationship between the degree of kidney injury and the concentrations of any plasma or urinary biomarkers. This indicates that any changes in biomarker levels did not closely reflect the amount of renal cell damage. However, uKIM-1 and pCys-C showed promise in potentially being able to detect histological AKI earlier than pCr. Histological

changes of Roundup toxicity have been reported previously (Sribanditmongkol et al., 2012; Williams et al., 2000). However, in our investigation, we identified specific modes of cell death in necrosis and apoptosis. These modes of cell death occurred in tubules and glomeruli during the acute stages of Roundup toxicity. We believe our description of Roundup-induced apoptosis is novel..

KIM-1 is a type I cell membrane glycoprotein expressed by tubule epithelial cells in response to injury. It appears in urine after proximal tubular injury (Ferguson et al., 2008; Hoffmann et al., 2010; Vaidya et al., 2008a). In this study, uKIM-1 outperformed other plasma and urinary biomarkers in early detection of AKI. However, as the specificity of this marker in predicting AKI is relatively low, there are limitations in terms of using uKIM-1 performance as an early biomarker in this animal model. Urinary KIM-1 gradually increased after Roundup® dosing and levels greater than 0.00029 µg/ml indicated pathological changes with high sensitivity (100%) but low specificity (58%). A highly sensitive cut-off point is useful to exclude the presence of nephrotoxicity, such that levels below this cut-off point have high negative predictive value. Our study showed that using 0.00029 µg/ml of uKIM-1 as a cut-off value could exclude histological changes with 100% accuracy; whereas 42% of those with a uKIM-1 over this threshold were false positives (no histological change). Both sensitivity and specificity are relevant to assessment of the usefulness of a diagnostic method (Bewick et al., 2004). ROC analysis can guide selection of the optimal cut-point for decision-making. To confirm a diagnosis, high specificity (> 95%) (Westin, 2001), or a high positive likelihood ratio is required (Bewick et al., 2004). Although a cut-off of over 0.00044 µg/ml showed a very high likelihood ratio (LR = 7), only 60% of animals that had kidney damage were detected.

However, at 24 h, pCr rose in the high dose group and remained high for 48 h. A concentration higher than 0.21 mg/dl was present in all cases with AKI. Although pCr concentration showed good performance (AUC-ROC of 0.89 at 24 h), the specificity for prediction of nephrotoxicity at 0.21 mg/dl was very low (44%), meaning 56% of those over this level were false positives. In other words, 56% of animals might have normal kidney histology and function. A cut-off point of > 0.28 mg/dl at 24 h gave the highest likelihood ratio (7.7), with 86% sensitivity and 90% specificity. It should be noted that Roundup® induced myocardial cell hypertrophy (Cha et al., 2000), which causes myotoxicity possibly leading to an increase of pCr independent of AKI.

In either case our findings provide some mechanistic support for using pCr to detect GPSH toxicity and to predict mortality in acute GPSH-poisoning in humans (Lee et al., 2008).

Some other biomarkers showed promise in AKI diagnosis including pCys-C, pNGAL and uVEGF. However, increases in these biomarkers were noted only at 24 h while pCr had already risen as early as 8 h after Roundup® dosing. Cys-C is a 13-kDa protein that is freely filtered via the glomerulus but reabsorbed and degraded in lysosomes of proximal tubule cells. It is not secreted by kidney tubules (Ferguson et al., 2008; Vaidya et al., 2008a). Recently, serum or pCys-C levels were found to perform better than pCr in some patients as a surrogate measure of kidney function (GFR) . In this present study, pCys-C levels and pCr levels rose in a similar time-dependent manner and appeared to be dose-dependent indicating that pCys-C could indicate kidney function, similar to pCr. Ideally, pCys-C is expected to increase earlier than pCr as it has a shorter half-life (Chiou and Hsu, 1975; Filler et al., 2005; Wyss and Kaddurah-Daouk, 2000). However, an increase in pCys-C levels was detected after pCr increased. This supports the idea of myotoxicity causing pCr increase.

NGAL is expressed and secreted by kidney epithelial cells (Ferguson et al., 2008; Hoffmann et al., 2010; Lisowska-Myjak, 2010; Vaidya et al., 2008a). According to the ROC analysis, neither urinary nor plasma NGAL levels were good early markers of AKI in our rat model, even though an increase in pNGAL levels was found after 24 h in rats treated with 2500 mg/kg of glyphosate. VEGF is an endothelial-specific growth factor involved in endothelial cell proliferation, differentiation and survival (Schrijvers et al., 2004). In rodent and human kidneys, VEGF is expressed predominantly in glomerular podocytes, distal tubules, and collecting ducts (Schrijvers et al., 2004). Therefore, uVEGF might be useful as an indicator for glomerular injury/dysfunction as this glycoprotein also regulates glomerular permeability (Breen, 2007; Schrijvers et al., 2004). The increase in uVEGF or uVEGF:Cr ratios could be a result of Roundup®-induced podocyte injury. Finally, AGP is another one of the markers elevated (uAGP:Cr ratio) after high dose Roundup® at 24 h in this study. This protein is an acute-phase glycoprotein produced primarily in the liver. An increase in AGP relates to several physiological states, for example; age, and pathological conditions including liver cirrhosis, nephritic disease

and acute inflammation (Bachtiar et al., 2009; Devarajan et al., 2010; Nickolas et al., 2008; Williams et al., 1997). Thus, in the present study APG elevation may not specifically indicate acute kidney damage.

The concentration of urinary biomarkers of AKI may be influenced by water reabsorption, urine flow rate and variation in urinary concentration within and between individuals (Ralib et al., 2012; Waikar et al., 2010). The ratio of biomarker over Cr (urine-Cr-normalised biomarker concentration) or the biomarker excretion rate has also been used for AKI prediction (Ilaria and Van, 1995; Ralib et al., 2012). GPSHs cause diarrhoea and gastrointestinal symptoms which can lead to dehydration and hypotension (Adam et al., 1997; Bradberry et al., 2004; Lee et al., 2008; Roberts et al., 2010). Even though normalised concentration slightly improved the statistical difference between poisoned rats and the time-matched controls, absolute concentration generally predicted AKI best, and the other two methods did not increase the ROC diagnostic performance. These results were similar to those presented in a previous study, a retrospective study of urinary biomarkers from the two-centre Early Intervention in Acute Renal Failure randomised controlled trail of high-dose erythropoietin for AKI prevention (Ralib et al., 2012).

Limitations to this study include: 1) While sCr/pCr and Cr clearance are generally accepted as reasonable surrogate markers of GFR, they are subject to measurement error and it is possible that some toxic substances may affect Cr production or distribution. The true change in GFR was not evaluated using a gold standard method such as inulin clearance. 2) Kidney dysfunction (for example, of drug transporter function in the proximal or distal tubule) could happen before histopathological change is detectable (Bonventre et al., 2010). 3) It should be noted that a commercial mixture of glyphosate and surfactants was used in this current study, and the surfactant used in Roundup® may contribute to herbicide nephrotoxicity (Seok et al., 2011). Ingestion of more than 8 ml of undiluted surfactant strongly related to AKI development. The frequency of critical AKI was 17% after ingestion of 137.6±139.5 ml glyphosate formulations (17-41% w/v of glyphosate) (Seok et al., 2011; Talbot et al., 1991).

In conclusion, we examined whether a panel of biomarkers and a traditional biomarker (pCr) can predict early kidney injury following Roundup® poisoning. The results showed a different time-course for each marker. By ROC analysis, uKIM-1

was the best biomarker at 8 h, predicting subsequent histological change in rats. However, the specificities of uKIM-1 and pCr performance for AKI prediction were relatively low. Plasma Cys-C mirrored kidney function in a similar way to pCr. However, pCr remains a worthwhile biomarker of early kidney injury and its usefulness should not be discounted in cases of nephrotoxicity.

Conflict of interest statement

There is no conflict of interest.

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Legends for Figures

Fig. 1. Histopathological changes in the kidneys of male rats following oral Roundup® administration, including necrotic cells and pyknotic nuclei (A), apoptotic cells (B) and total cell death (C). Each data point represents the mean ± SD of 6 rats. The means were calculated from average numbers of necrotic cells or apoptotic cells found in the cortex and medulla. Statistically significant changes from controls are indicated by * $p < 0.05$ and ** $p < 0.01$. Light micrographs of the kidneys from control (D) and Roundup® groups (E,F) stained with hematoxylin-eosin. Nuclei of glomeruli (arrowhead) were often pyknotic (E). Areas containing necrosis (arrowhead) are visible in the hematoxylin-eosin stain in Roundup®-treated sections (F). Tubular cells contain apoptotic nuclei (arrowhead) are visible in the Apoptag stain in Roundup®-treated sections (G). Each bar indicates 100 μm.

Fig. 2 Correlation (r) between apparent clearances of neutrophil gelatinase-associated lipocalin (A), cystatin-C (B) and creatinine clearance (Cr). Relationships between plasma cystatin-C and plasma creatinine (C), plasma cystatin-C and creatinine clearance (D).

Fig. 3 Time-course of changes in injury biomarker concentrations. Urinary kidney injury molecule-1 (A), plasma neutrophil gelatinase-associated lipocalin (B), urinary clusterin (C) plasma cystatin-C (D), urinary vascular endothelial growth factor (E) and plasma creatinine (F). The grey areas indicate normal ranges. The error bars represent IQR (n=4).

Fig. 4 AUCs for the absolute urinary concentration, normalised urinary concentration, and urinary excretion rate. The mean AUCs with upper and lower 95% CI are presented. for urinary β_2-microglobulin (A), cystatin-C (B), albumin (C), clusterin (D), osteopontin (E), kidney injury molecule-1 (F), neutrophil gelatinase-associated lipocalin (G). * $p < 0.05$ for the difference between the AUC of normalised concentration or excretion rate and the absolute concentration.

Table 1 Levels of plasma and urinary renal biomarkers

Biomarkers	Dose (mg/kg)	0-8 h	8-24 h	24-48 h
pNGAL (µg/ml)	Control	0.06±0.03	0.06±0.02	0.10±0.10
	250	0.06±0.01	0.20±0.26	0.08±0.02
	500	0.08±0.01	0.08±0.05	0.07±0.06
	1200	0.09±0.03	0.08±0.05	0.05±0.04
	2500	0.08±0.02	0.39±0.33***	0.53±0.20**
pCys-C (µg/ml)	Control	1.51±0.73	1.64±0.49	2.21±0.26
	250	1.17±0.08	1.64±0.09	2.20±0.75
	500	1.43±0.04	1.01±0.59	1.38±0.94
	1200	1.41±0.14	1.57±0.40	2.26±2.51
	2500	0.86±0.61	3.89±0.81***	4.18±1.18
pCr (mg/dl)	Control	0.23±0.06	0.23±0.06	0.22±0.07
	250	0.21±0.02	0.27±0.04	0.25±0.11
	500	0.22±0.02	0.30±0.05*	0.32±0.18*
	1200	0.32±0.06	0.29±0.07	0.32±0.18
	2500	0.35±0.06*	0.58±0.08*	0.44±0.18**
uKIM-1 (ng/ml)	Control	0.44±0.24	0.30±0.09	0.29±0.12
	250	0.37±0.04	0.42±0.12	0.56±0.59
	500	0.40±0.10	0.49±0.33	0.92±0.32**
	1200	0.62±0.15	0.76±0.65	0.29±0.13
	2500	0.82±0.38	0.48±0.22	1.45±1.17**
uVEGF (ng/ml)	Control	0.34±0.15	0.20±0.01	0.22±0.10
	250	0.26±0.08	0.17±0.06	0.12±0.08
	500	-	-	-
	1200	0.12±0.15	0.23±0.06	0.14±0.12
	2500	0.60±0.36	4.07±1.60**	2.83±1.75**

Statistically significant changes comparing to the controls are indicated by *$p < 0.05$

and ** $p < 0.01$. p = plasma; u = urine; Cr = creatinine KIM-1 = kidney injury

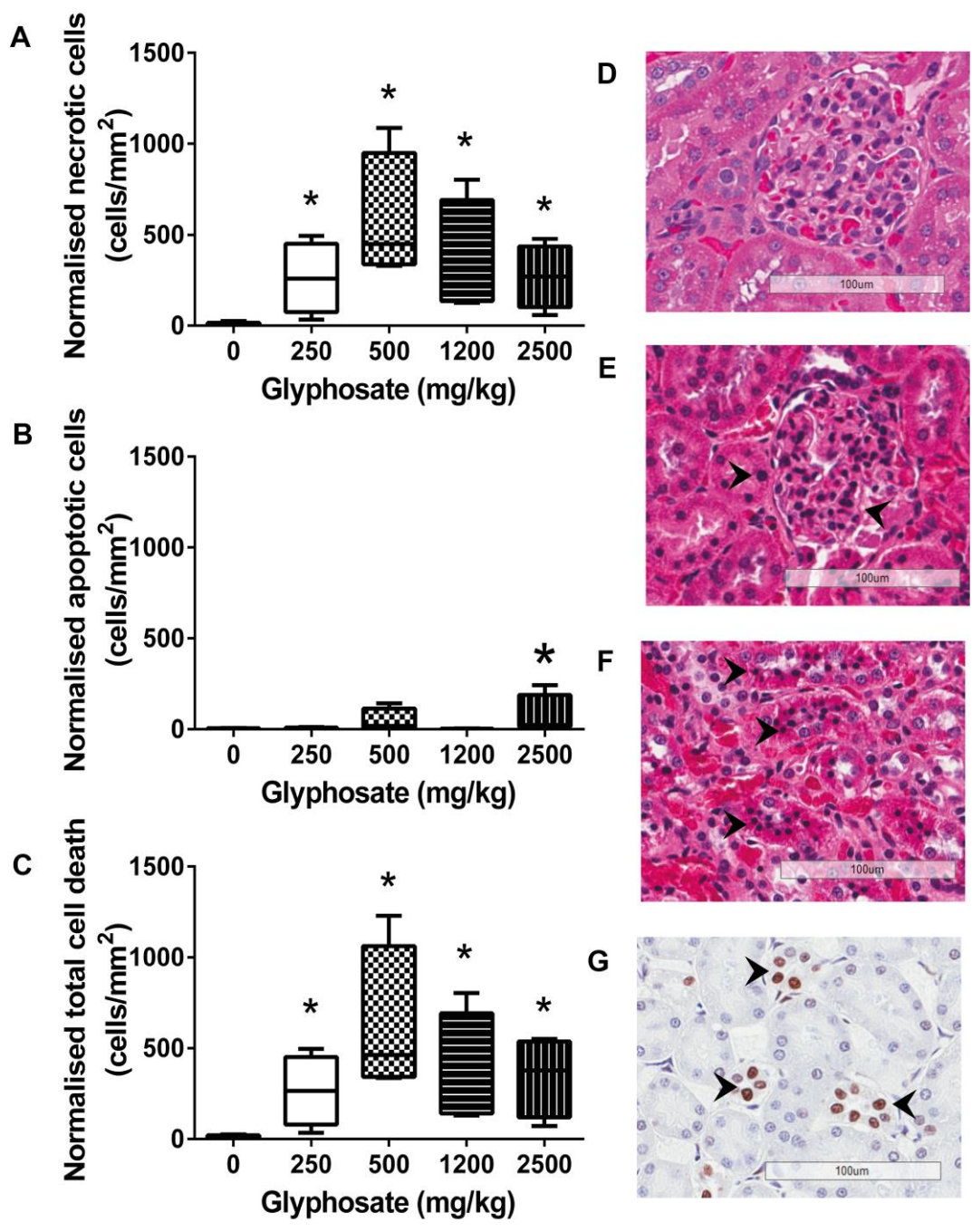molecule-1; Cys-C = cystatin-C; NGAL = neutrophil gelatinase-associated lipocalin;

VEGF = vascular endothelial-specific growth factor.

Table 2 Performance of absolute concentration biomarkers for prediction of AKI .

Time-point	Marker	AUC	Sensitivity (%)	Specificity (%)	Likelihood ratio	Cut-off
8 h	uKIM-1	0.85	100	58	2.4	> 0.00029 µg/ml
	uKIM-1	0.85	92	66	2.8	> 0.00033 µg/ml
	uKIM-1	0.85	60	92	7.0	> 0.00044 µg/ml
24 h	pCr	0.89	100	44	1.8	> 0.21 mg/dl
	pCr	0.89	70	90	6.2	> 0.29 mg/dl
	pCr	0.89	63	100	-	> 0.31 mg/dl
48 h	pCr	0.96	100	67	3	> 0.23 mg/dl
	pCr	0.96	86	90	7.7	> 0.28 mg/dl
	pCr	0.96	78	100	-	> 0.29 mg/dl
	pCys-C	0.80	100	39	1.6	> 1.5 µg/ml
	pCys-C	0.80	79	61	2	> 1.7 µg/ml
	pCys-C	0.80	43	96	12	> 2.5 µg/ml
	pNGAL	0.78	100	25	1.3	> 4.2 µg/ml
	pNGAL	0.78	78	60	2	> 6.8 µg/ml
	pNGAL	0.78	60	96	16	> 9.2 µg/ml

AUC = area under the curve; p = plasma; u = urine; Cr = creatinine KIM-1 = kidney injury molecule-1; Cys-C = cystatin-C; NGAL = neutrophil gelatinase-associated lipocalin
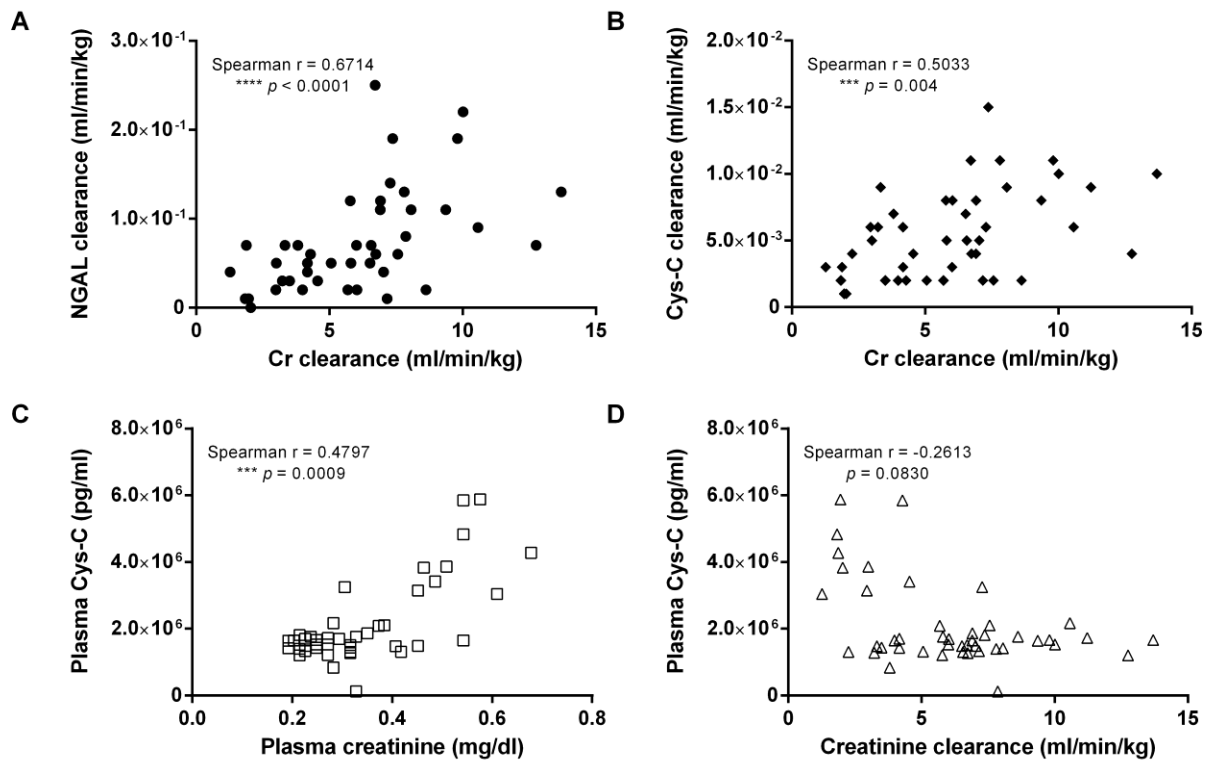
Fig. 1

Fig. 2

A

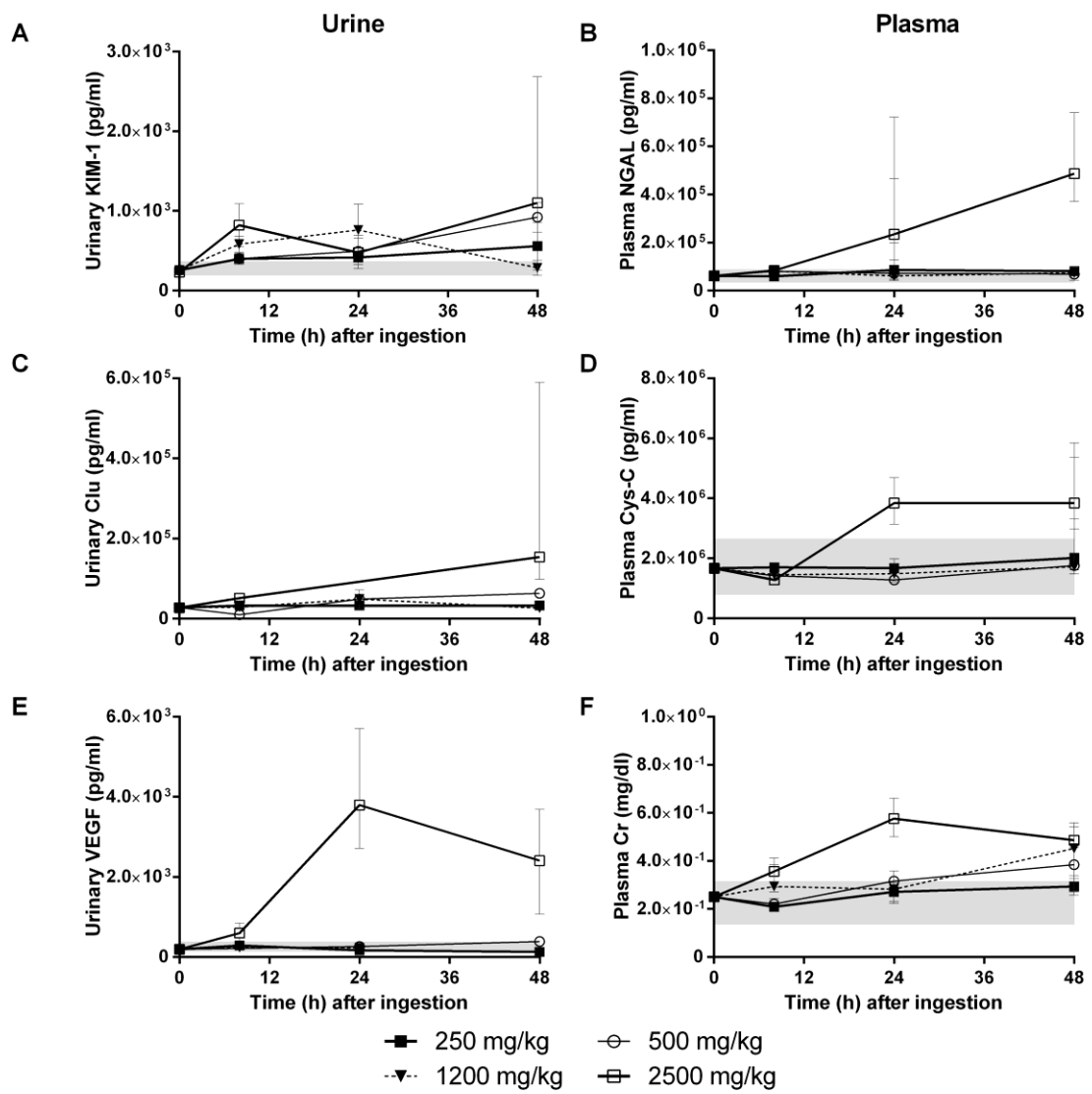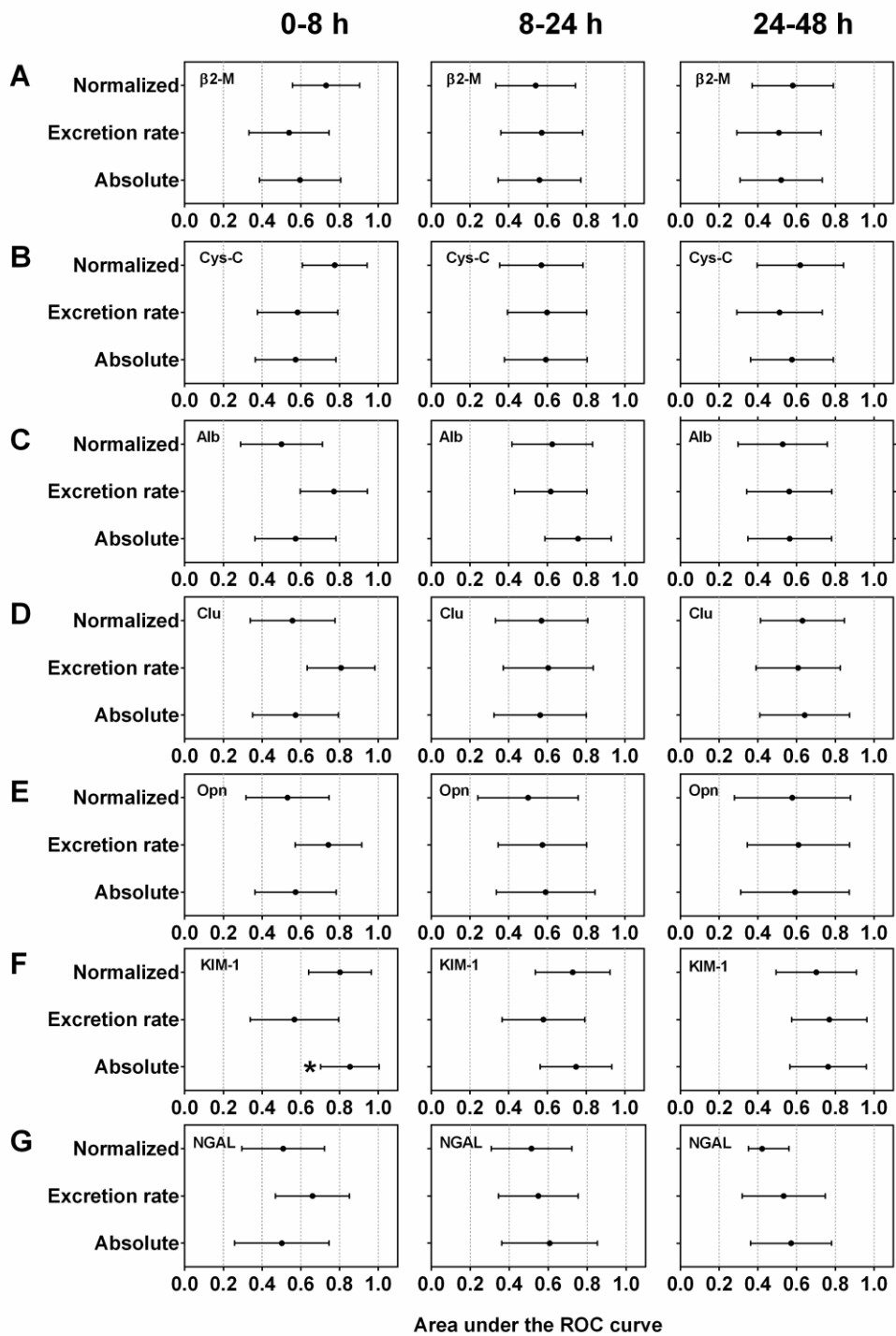Spearman r = 0.6714
**** *p* < 0.0001

NGAL clearance (ml/min/kg) vs Cr clearance (ml/min/kg)

B

Spearman r = 0.5033
*** *p* = 0.004

Cys-C clearance (ml/min/kg) vs Cr clearance (ml/min/kg)

C

Spearman r = 0.4797
*** *p* = 0.0009

Plasma Cys-C (pg/ml) vs Plasma creatinine (mg/dl)

D

Spearman r = -0.2613
p = 0.0830

Plasma Cys-C (pg/ml) vs Creatinine clearance (ml/min/kg)

22

Fig. 3

A Urine

Urinary KIM-1 (pg/ml)

B Plasma

Plasma NGAL (pg/ml)

C

Urinary Clu (pg/ml)

D

Plasma Cys-C (pg/ml)

E

Urinary VEGF (pg/ml)

F

Plasma Cr (mg/dl)

Time (h) after ingestion

■ 250 mg/kg   ○ 500 mg/kg
▼ 1200 mg/kg   ⊟ 2500 mg/kg
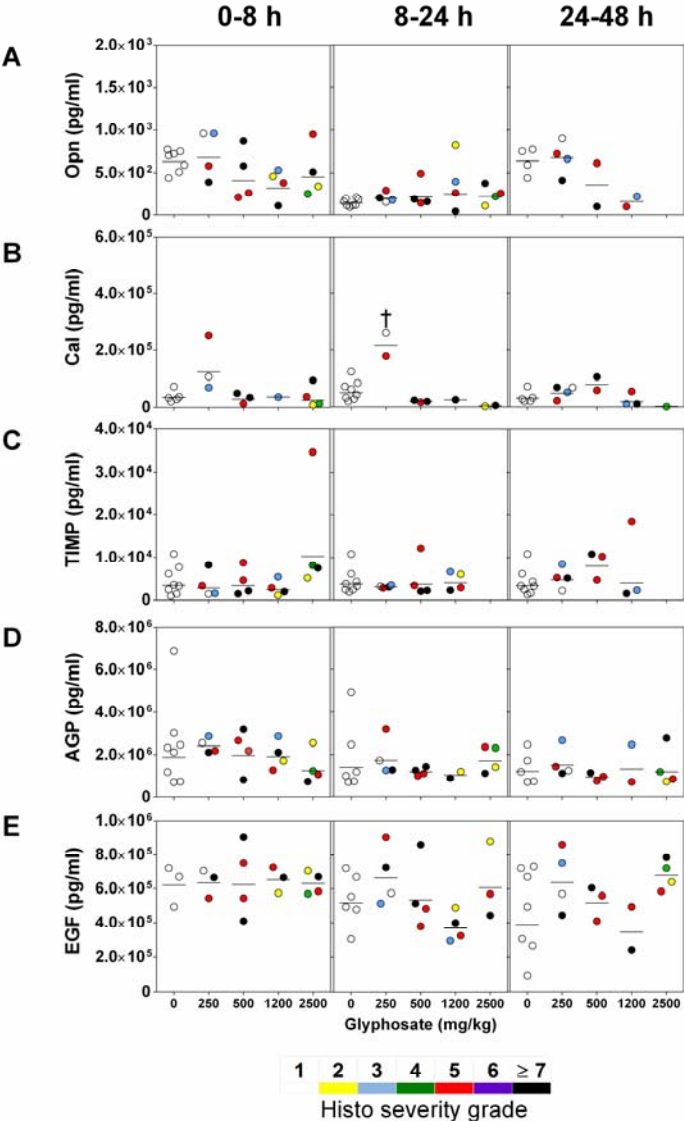
23

Fig 4

ROC performance:Roundup® nephrotoxicity
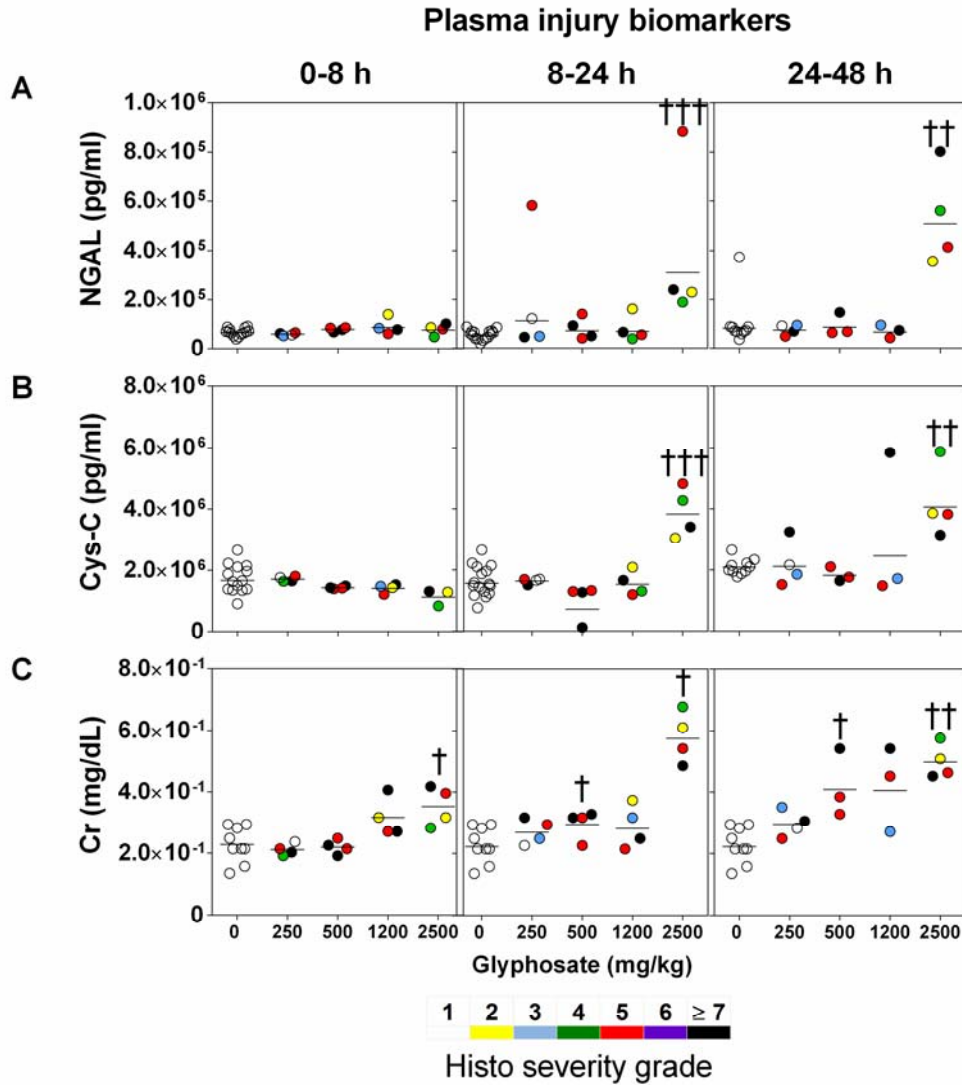
Supplementary Fig. 1. Plasma injury markers. Levels of plasma neutrophil gelatinase-associated lipocalin (A), plasma cystatin-C (B) and plasma creatinine (C) were measured in rats. Roundup® was administered orally at glyphosate doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. The severity grades of necrotic cells and pyknotic nuclei after Roundup® treatment are indicated on a scale of 1 (normal) to ≥7 with indicated grades displayed as the following colours: grade 1 (white), grade 2 (yellow), grade 3 (blue), grade 4 (green), grade 5 (red), grade 6 (purple) and grade ≥7 (black). The line indicates the mean for each group. Statistically significant changes are indicated by † $p < 0.05$ and †† $p < 0.01$.
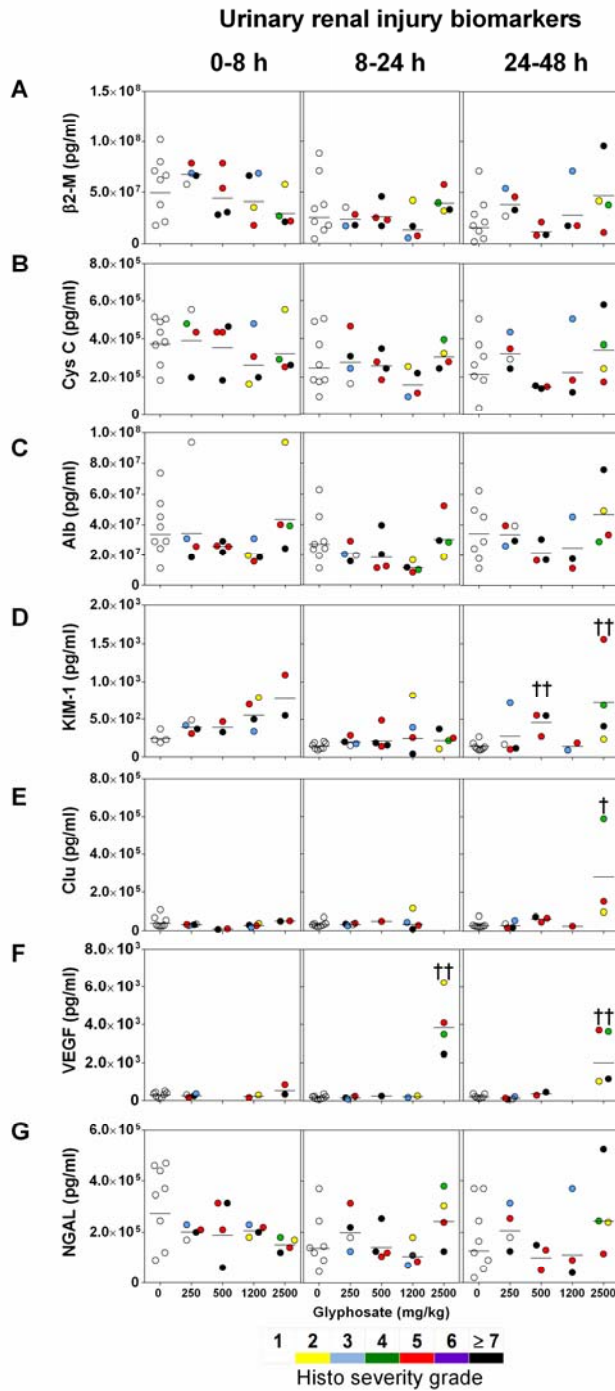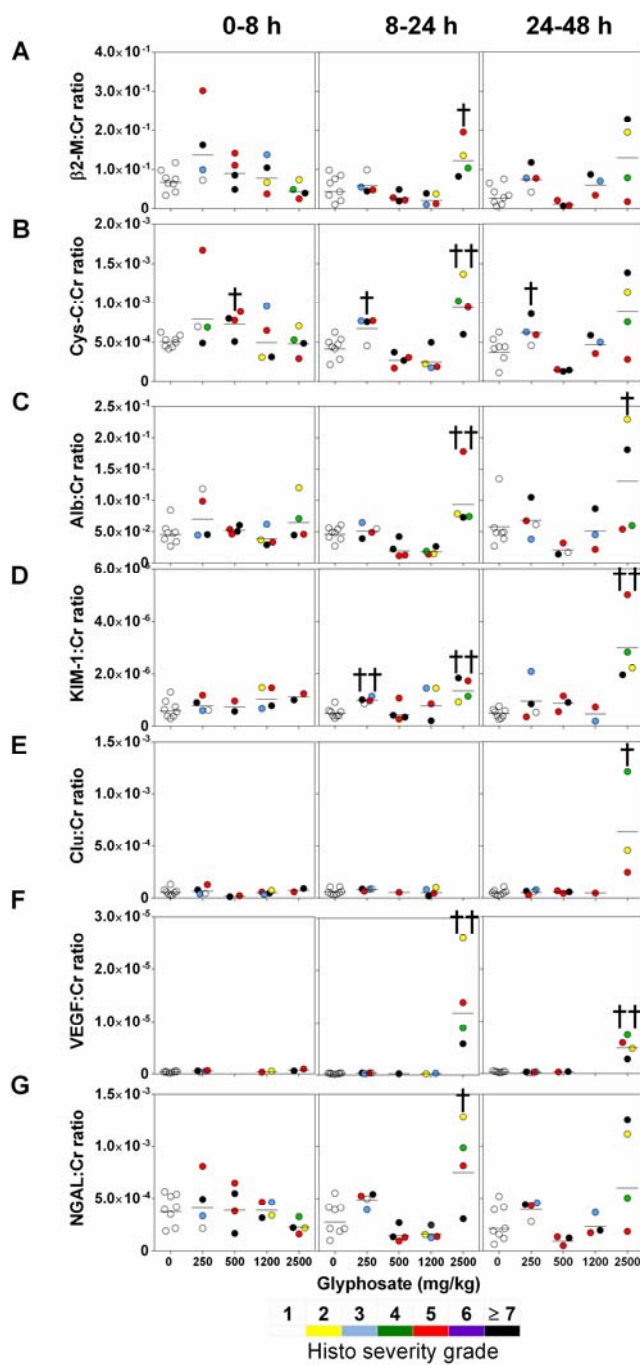
Supplementary Fig. 2 Urinary kidney injury biomarkers. Levels of urinary β_2-microglobulin (A), urinary cystatin-C (B), urinary albumin (C), urinary kidney injury molecule-1 (D), urinary clusterin (E), urinary vascular endothelial growth factor (F) and urinary neutrophil gelatinase-associated lipocalin (G) were measured in rats. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.
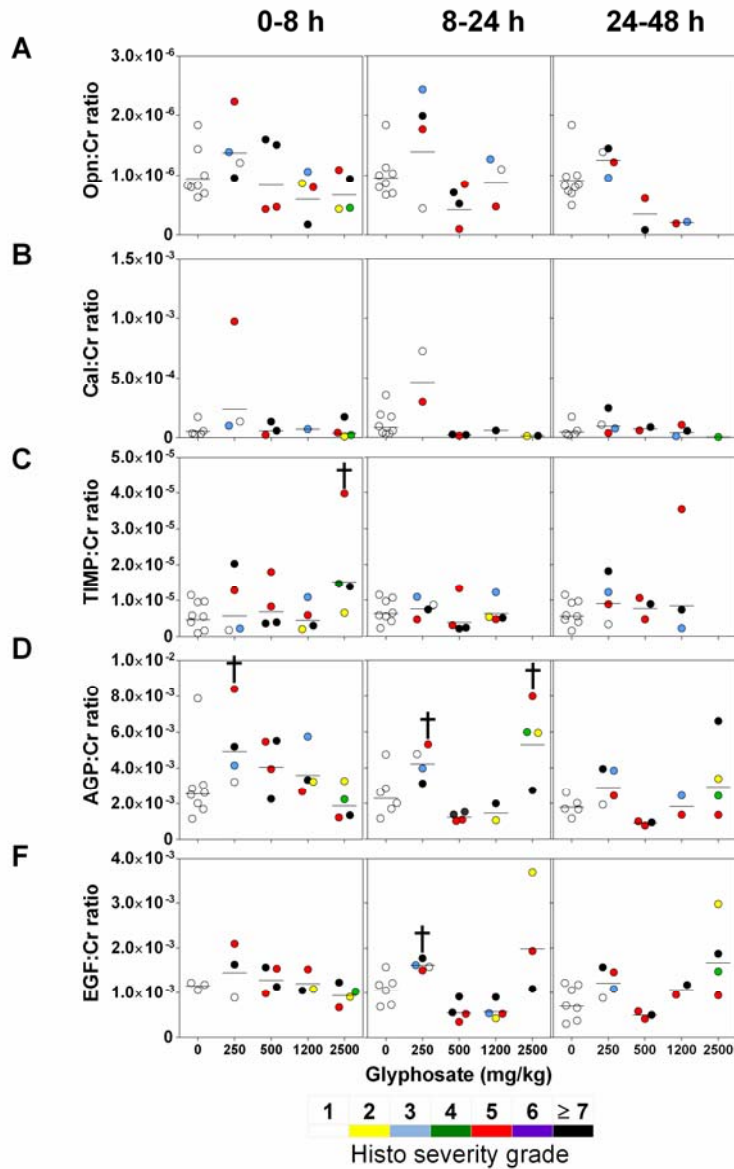
Supplementary Figure 3

Urinary kidney injury biomarkers. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Osteopontin (A), Calbindin-D28 (B), tissue inhibitor of metalloproteinases (C), α-1-acid glycoprotein (D), epidermal growth factor (F). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.
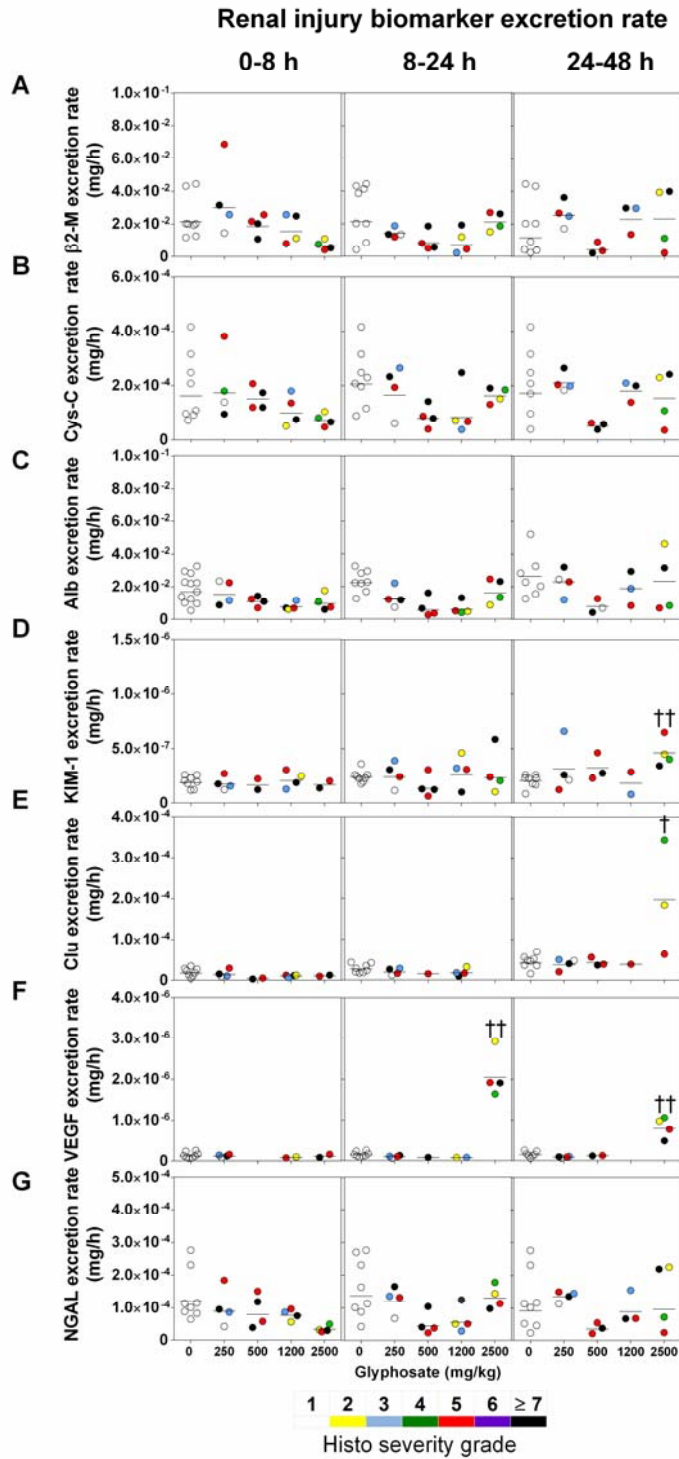
Supplementary Figure 4

Normalisation of biomarkers to urine creatinine concentration. Ratios of urinary β_2- macroglobulin to creatinine (A), urinary cystatin-C to creatinine (B), urinary albumin to creatinine (C), urinary kidney injury molecule-1 to creatinine (D), urinary clusterin to creatinine (E), urinary vascular endothelial growth factor to creatinine (F) and urinary neutrophil gelatinase-associated lipocalin to creatinine (G) were measured in rats. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.

Supplementary Figure 5

Normalization to urine creatinine. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Osteopontin (A), Calbindin-D28 (B), tissue inhibitor of metalloproteinases (C), α-1-acid glycoprotein (D), epidermal growth factor (F). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.
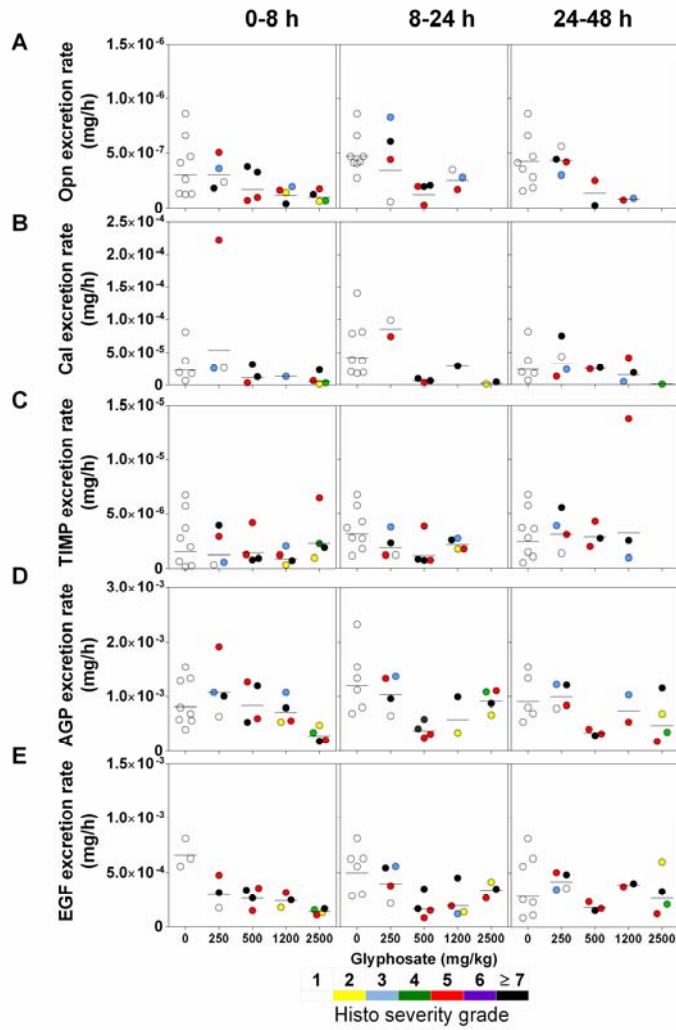
Supplementary Figure 6 Biomarker excretion rate. Excretion rate of urinary β2- macroglobulin (A), urinary cystatin-C (B), urinary albumin (C), urinary kidney injury molecule-1 (D), urinary clusterin (E), urinary vascular endothelial growth factor (F) and urinary neutrophil gelatinase-associated lipocalin (G) were measured in rats. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.
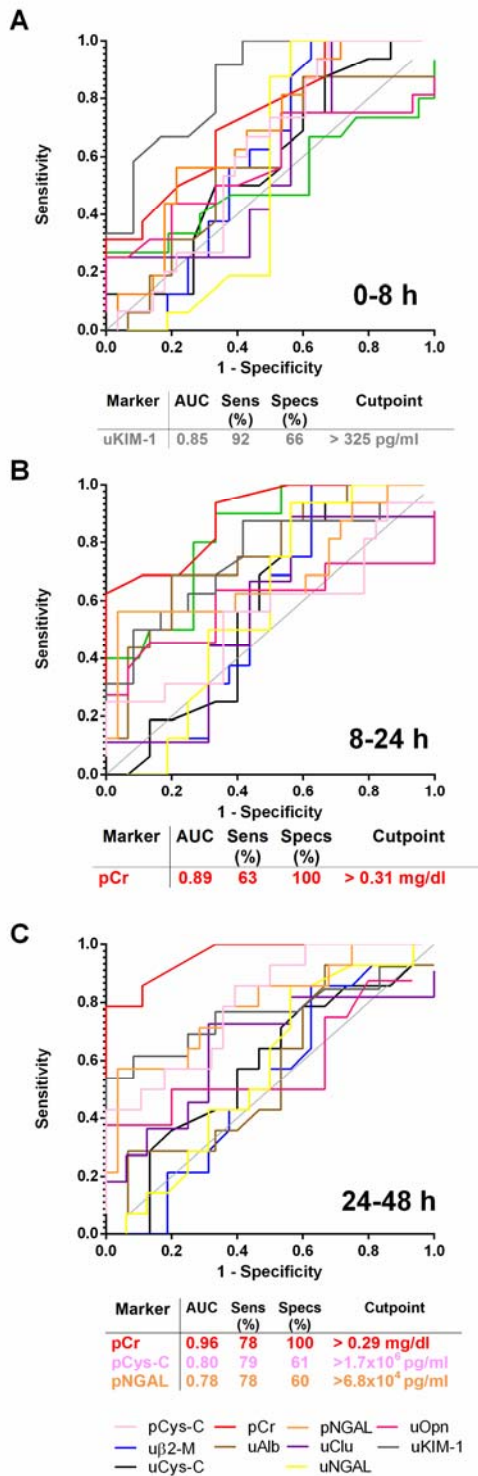
Supplementary Figure 7

Biomarker excretion rate. Roundup® was administered orally at doses of 0 or 250 or 500 or 1200 or 2500 mg/kg to groups of 10 rats (zero/control group) and 4 rats (treatment groups). Osteopontin (A), Calbindin-D28 (B), tissue inhibitor of metalloproteinases (C), α-1-acid glycoprotein (D), epidermal growth factor (F). Time is indicated on the top and dose is indicated at the bottom. Circles indicate biomarker values from individual animals. Kidney histopathological assessment was performed at 48 h. Severity grades and statistically significant changes are as described under Supplementary Figure 1.

Absolute concentration performance

Marker	AUC	Sens (%)	Specs (%)	Cutpoint
uKIM-1	0.85	92	66	> 325 pg/ml

Marker	AUC	Sens (%)	Specs (%)	Cutpoint
pCr	0.89	63	100	> 0.31 mg/dl

Marker	AUC	Sens (%)	Specs (%)	Cutpoint
pCr	0.96	78	100	> 0.29 mg/dl
pCys-C	0.80	79	61	>1.7x10^6 pg/ml
pNGAL	0.78	78	60	>6.8x10^4 pg/ml

Legend: pCys-C, pCr, pNGAL, uOpn, uβ2-M, uAlb, uClu, uKIM-1, uCys-C, uNGAL

Supplementary Fig. 8 ROC curves for absolute concentration biomarkers at 0-8 h (A) 8-24 h (B) and 24-48 h (C) with respect to a composite histopathology score for necrotic cells and pyknotic nuclei.