Subclinical chronic kidney disease modifies the diagnosis of experimental acute kidney injury

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Extensive structural damage within the kidney must be present before serum creatinine increases. However, a subclinical phase of chronic kidney disease (CKD) usually goes undetected. Here we tested whether experimental subclinical CKD would modify functional and damage biomarker profiles of acute kidney injury (AKI). Subclinical CKD was induced in rats by adenine or aristolochic acid models but without increasing serum creatinine. After prolonged recovery (three to six weeks), AKI was induced with a subnephrotoxic dose of cisplatin. Urinary levels of kidney injury molecule-1 (KIM-1), cytochrome C, monocyte chemotactic protein-1 (MCP-1), clusterin, and interleukin-18 increased during CKD induction, without an increase in serum creatinine. After AKI in adenine-induced CKD, serum creatinine increased more rapidly, while increased urinary KIM-1, clusterin, and MCP-1 were delayed and reduced. Increased serum creatinine and biomarker excretion were associated with diffuse tubulointerstitial injury in the outer stripe of outer medulla coupled with over 50% cortical damage. Following AKI in aristolochic acid–induced CKD, increased serum creatinine, urinary KIM-1, clusterin, MCP-1, cytochrome C, and interleukin-18 concentrations and excretion were greater at day 21 than day 42 and inversely correlated with cortical injury. Subclinical CKD modified functional and damage biomarker profiles in diametrically opposite ways. Functional biomarker profiles were more sensitive, while damage biomarker diagnostic thresholds and increases were diminished and delayed. Damage biomarker concentrations and excretion were inversely linked to the extent of prior cortical damage. Thus, thresholds for AKI biomarkers may need to be lower or sampling delayed in the known presence of CKD.

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There is a global epidemic of chronic kidney disease (CKD), with 470 million CKD cases and a prevalence in Western populations of 10% to 16%.[1,2] CKD is a strong independent risk factor for cardiovascular mortality[3] and acute kidney injury (AKI).[4] In turn, AKI is strongly linked to the development of new CKD, progression of CKD, increased risk of end-stage kidney disease (ESKD), and short- and long-term mortality.[5,6] Thus, AKI and CKD are interconnected syndromes.[4] Unfortunately, kidney structure and function are poorly correlated. In diseased kidneys, interstitial fibrosis and nephron loss may correlate with glomerular filtration rate (GFR),[7] although no correlation with interstitial fibrosis has been observed in some studies.[8] This begs the question of how to detect progressive kidney damage before it becomes overt and irreversible.

CKD is defined by a persistent estimated GFR (eGFR) of <60 ml/min/1.73 m^2, as calculated from serum creatinine (sCr) level. Therefore, subclinical CKD, which must precede overt CKD, may be defined as extensive kidney damage in the absence of increased sCr. Because an increase in sCr is not detected until eGFR is reduced to approximately 50 to 60 ml/min/1.73 m^2, this suggests that AKI occurs frequently on a background of subclinical CKD, even when baseline sCr is in the normal range.

Subclinical CKD cannot currently be recognized without direct measurement of GFR or renal biopsy. Neither measurement is usually undertaken in patients with a normal sCr. We hypothesized that biomarkers of damage could provide a method of detecting background injury in patients with an apparently normal sCr. Because stratification for CKD improved biomarker performance in studies of AKI,[9,10] we anticipated that subclinical CKD would similarly alter biomarker performance in diagnosis of AKI. In particular, we hypothesized that damage biomarker concentrations would be inversely associated with the extent of prior renal damage. We used 2 rodent models of subclinical CKD to investigate the performance of a panel of urinary biomarkers after AKI was superimposed using the established nephrotoxin-induced cisplatin (cis) model.[11] Subclinical CKD was induced with adenine or aristolochic acid (AA). Both rodent models produce changes in kidney structure and function that mimic human CKD, including tubulointerstitial damage (TID) characterized by inflammation, atrophy, and fibrosis, but via differing mechanisms.[12–14] After an adenine-supplemented diet, diffuse structural TID develops in the outer stripe of

the outer medulla (OSOM), with focal lesions in the cortex.[15] Adenine injures tubular epithelial cells by hyuricemic and perhaps direct oxidative stress and promotes infiltration of inflammatory cells and fibrosis.[15] In AA-induced CKD, daily i.p. AA-1 (the active ingredient in AA) for 5 days causes DNA adduct formation within tubular epithelial cells, followed by cellular apoptosis and inflammation beginning in the OSOM and progressing to the cortex.

Subclinical CKD models were created by limiting the dose and duration of adenine and AA so that sCr did not increase during CKD induction, and by providing prolonged (4 weeks for adenine-treated and 3 or 6 weeks for AA-treated rats) recovery before superimposing AKI. A further refinement was choosing a low (2 mg/kg) dose of cis to induce AKI that was subnephrotoxic in control kidneys, defined as producing histological damage without an increase in sCr. Temporal profiles of urinary levels for kidney injury molecule 1 ($_u$KIM-1), cytochrome C ($_u$cytochrome C), monocyte chemotactic protein 1 ($_u$MCP-1), $_u$clusterin, interleukin 18 ($_u$IL-18), and osteopontin were monitored in both models before and after induction of AKI.

RESULTS

Kidney function

During the induction and recovery phases of adenine- and AA-induced CKD, sCr remained effectively unchanged (Figure 1), with minor increases in sCr on days 21, 28, and 56 (Figure 1a), but at low levels (below 40 μmol/l, a level deemed significant for renal damage in rats).[16] In AA-induced CKD (Figure 1c and d), sCr remained at basal levels from induction until day 21 (34.04 ± 3.87 μmol/l) or day 42 (30.60 ± 4.18 μmol/l).

After subnephrotoxic cis (2 mg/kg) on day 56, there was no increase in sCr in controls, but sCr increased rapidly in both subclinical CKD models. In AA-induced CKD, greater sCr increases occurred with earlier (21-day) than later (42-day) cis (Figure 1c and e). After nephrotoxic cis (4 mg/kg), sCr increased more rapidly in adenine-treated rats than controls, peaking at day 57 (vs. day 59). Decreases in creatinine clearance mirrored increases in sCr in both CKD models (Figure 1b, d, and f).

Urinary biomarkers

Urinary biomarker profiles (Figures 2–5) differed depending on the presence and extent of prior CKD pathology, dose of cis in adenine-induced CKD, and duration of recovery in AA-induced CKD.

KIM-1

During induction of CKD, $_u$KIM-1 concentrations increased immediately in both models. After adenine, these remained elevated during supplementation, returning to baseline (zero) during recovery (Figure 2a) despite the presence of extensive OSOM and cortical injury (see below). In AA-induced CKD, $_u$KIM-1 had returned to baseline by day 5 (Figure 3a and b).

In adenine-induced CKD both subnephrotoxic (2 mg/kg) and nephrotoxic (4 mg/kg) cis resulted in immediate increases in $_u$KIM-1 (Figure 2a and Supplementary Table S1). However, $_u$KIM-1 increases were delayed and reduced in magnitude compared with control rats at the same dose of cis. One week after cis (day 63), similar levels were detected in the adenine and control groups administered cis. Total excretion of KIM-1 over 72 hours after injury mirrored increases in serum levels with reduced excretion in the presence of subclinical CKD versus controls after the same cis dose (Figure 4a and b).

In AA-CKD, $_u$KIM-1 increases were much greater than in controls when cis was administered on day 21 but lower and delayed or comparable to controls after administration on day 42 (Figure 3a and b; Supplementary Table S2). $_u$KIM-1 increases were similar in controls at both time points. The much greater 72-hour urinary excretion of KIM-1 after cis on day 21 versus day 42 (Figure 5a and b) was consistent with the different temporal urinary concentration profiles.

After both 2 and 4 mg/kg cis, at least 75% of the cortex was injured in adenine-induced CKD, with even more involvement of the OSOM (Figures 4 and 6b). Total KIM-1 excretion over 72 hours after cis was increased in proportion to cortical injury in both adenine-induced CKD (r^2 = 0.70 and 0.74, respectively, for 2 mg/kg and 4 mg/kg cis; Supplementary Table S3) and AA-induced CKD (r^2 = 0.6 and 0.76, respectively, for days 21 and 42; Supplementary Table S3). The increase in cortical histological injury in adenine-induced CKD was similar and approximately 50% higher than baseline after both 2 mg/kg and 4 mg/kg cis (Figure 6b), and in AA-induced CKD about 50% after 2 mg/kg cis on day 21 (82%) but somewhat less after injury on day 42 (54%) (Figure 6c).

Other biomarkers

In general, the temporal profiles of the other damage biomarkers were similar to those observed for KIM-1, with an immediate increase during induction of CKD with adenine or AA and eventual return to baseline before superimposition of AKI. Urinary osteopontin concentrations remained unchanged in both AA-induced CKD (not shown) and adenine-induced CKD (Figure 2f) irrespective of cis dose and will not be discussed further.

In adenine-induced CKD, the increases in clusterin and MCP-1 after 4 mg/kg cis were similarly reduced and/or delayed compared with controls, although differentiation was not present at the lower cis dose (Figure 2b and c; Supplementary Table S1). This pattern reversed for $_u$IL-18, with levels higher in adenine-induced CKD than controls after 2 mg/kg cis (Supplementary Table S1), possibly suggesting selective activation of this biomarker with adenine. No differentiation between biomarker increases in adenine CKD and controls was apparent for cytochrome C (Figure 2d and Supplementary Table S1).

In AA-induced CKD, increases in urinary clusterin and to some extent MCP-1 mimicked KIM-1 as described, with

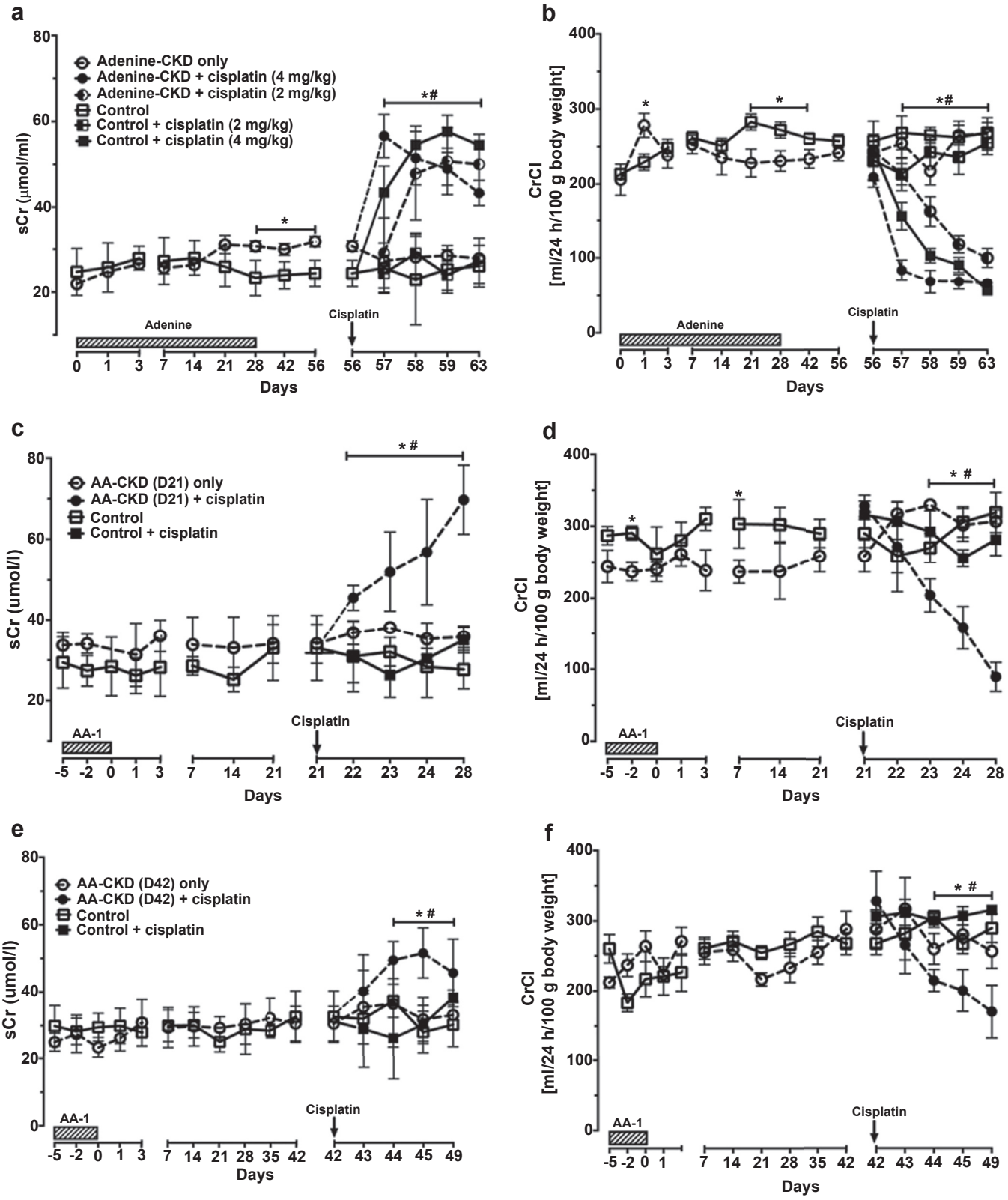

Figure 1 | Acute kidney injury superimposed on adenine- and aristolochic acid–induced chronic kidney disease (AA-CKD): serum creatinine (sCr) and creatinine clearance. (**a,c,e**) sCr remained effectively at baseline in adenine- and AA-induced CKD rats. Cisplatin was administered after 4 weeks' recovery in adenine-induced CKD (Adenine-CKD) at a subnephrotoxic (2 mg/kg) or nephrotoxic (4 mg/kg) dose and at either 21 days or 42 days in AA-induced CKD (2 mg/kg at each). (**b,d,f**) Changes in creatinine clearance inversely mirrored sCr. Data are expressed as mean ± SD; significance: $^{*}P < 0.05$ versus controls and $^{\#}P < 0.05$ versus baseline.

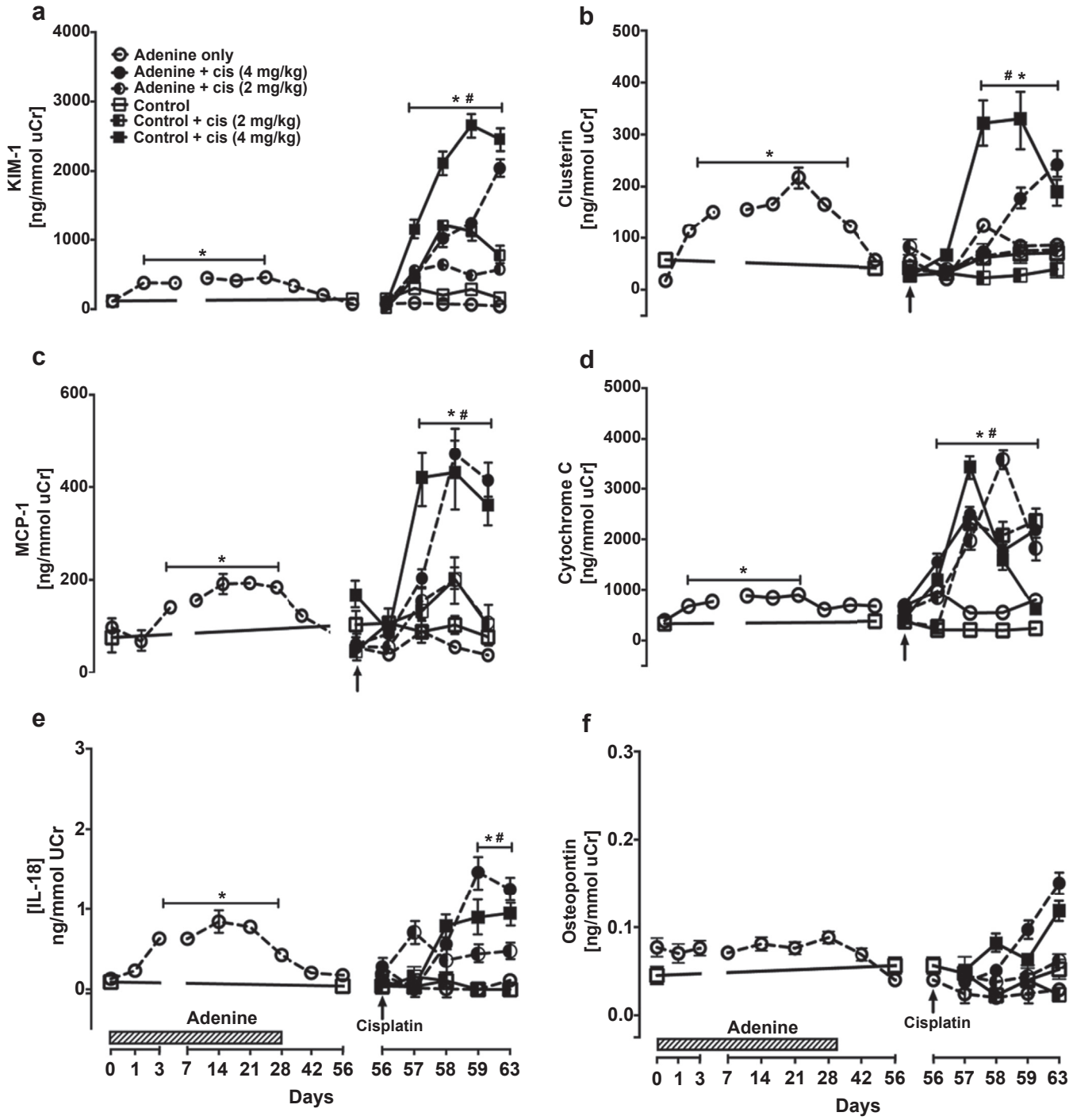

Figure 2 | Acute kidney injury superimposed on adenine-induced chronic kidney disease (Adenine-CKD): damage biomarker profiles. The panels show profiles for (**a**) KIM-1, (**b**) Clusterin, (**c**) MCP-1, (**d**) cytochrome C, (**e**) IL-18, and (**f**) osteopontin. All biomarkers except osteopontin increased during induction of CKD with adenine (days 0 to 21) and returned to baseline by day 56. Both subnephrotoxic (2 mg/kg) and nephrotoxic (4 mg/kg) cisplatin produced immediate increases in most biomarkers ($_u$KIM-1, $_u$clusterin, $_u$MCP-1, $_u$cytochrome C, and $_u$IL-18). However, biomarker increases were delayed and reduced in magnitude compared with controls. Data are normalized to urinary creatinine concentration and shown as mean ± SEM; significance: $^*P < 0.05$ versus controls and $^{\#}P < 0.05$ versus baseline.

more marked increases than in controls after cis at day 21 and with reduced concentrations when AKI was imposed on day 42 (Figure 3c and e; Supplementary Table S2). In addition, the urinary profiles of clusterin, MCP-1, cytochrome C, and IL-18 were similarly reduced and delayed in AA-induced CKD at day 42 when compared with controls. Consequently, temporal biomarker profiles at day 42 in AA-induced CKD were similar to the key observation when AKI was induced in adenine-induced CKD, namely that concentrations were reduced and delayed in the presence of subclinical CKD with

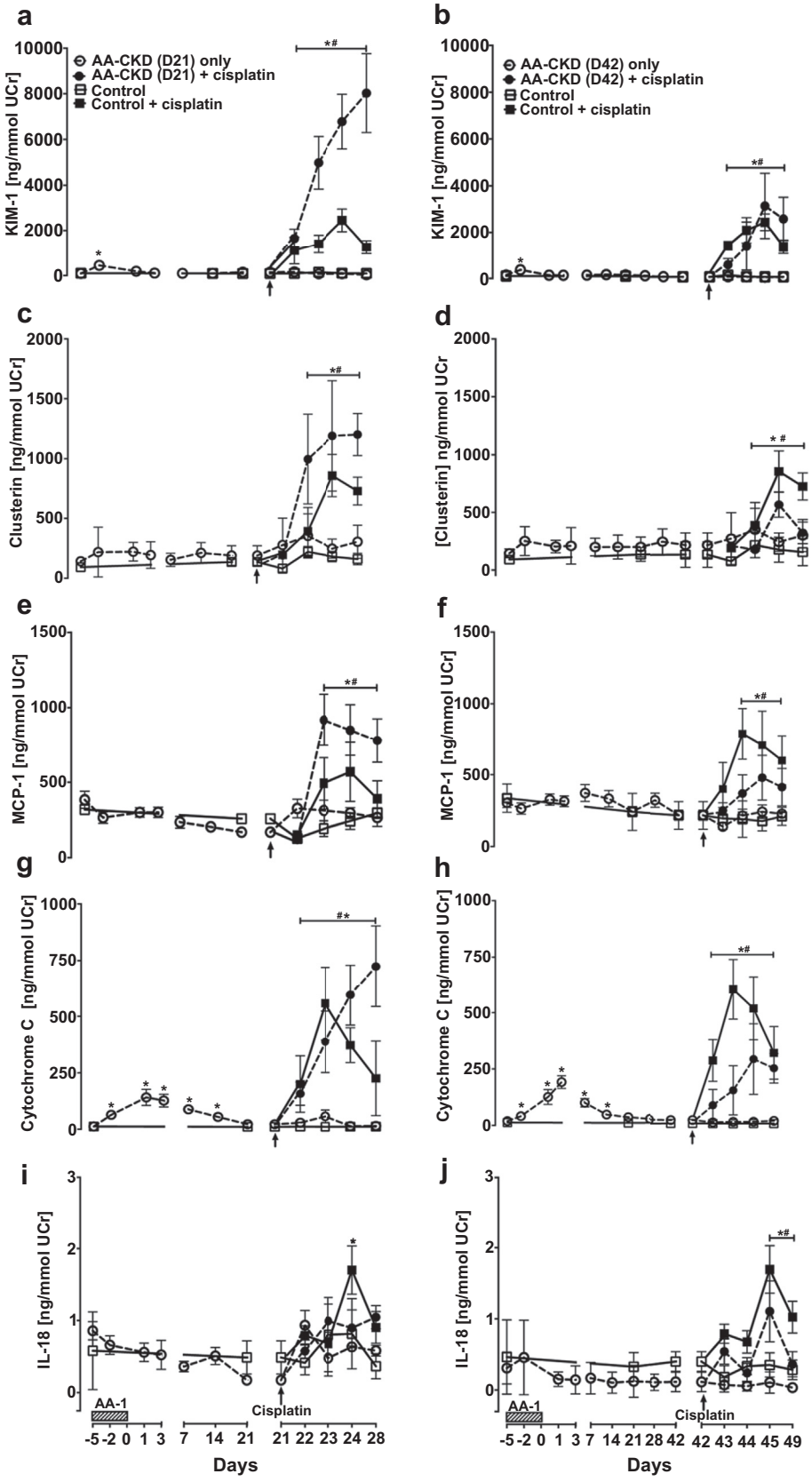

Figure 3 | Acute kidney injury superimposed on aristolochic acid–induced chronic kidney disease (AA-CKD): damage biomarker profiles. Increases in $_u$KIM-1 (**a,b**), $_u$clusterin (**c,d**), uMCP-1 (**e,f**), and $_u$cytochrome C (**g,h**), and to some extent $_u$IL-18 (**i,j**) were greater than in controls when cisplatin was given on day 21 but lower and delayed or comparable to controls after more prolonged recovery and administration on day 42. Data are expressed as mean ± SD; significance: $^*P < 0.05$ for values versus controls and $^{\#}P < 0.05$ versus baseline.

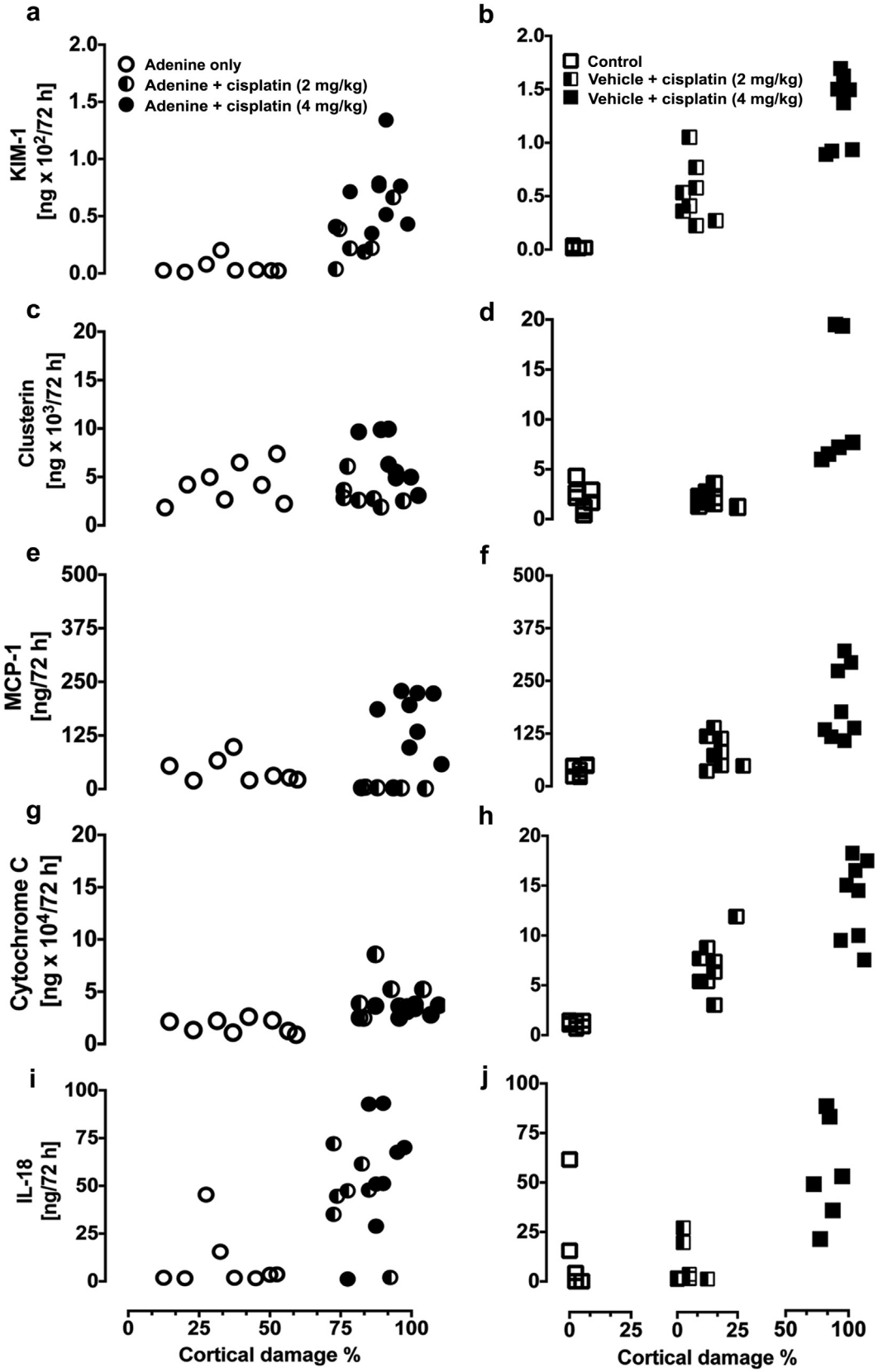

Figure 4 | Biomarker excretion versus cortical damage after acute kidney injury in adenine-induced chronic kidney disease (CKD) and controls. Total biomarker excretion for 72 hours after cisplatin administration for KIM-1 (**a,b**), clusterin (**c,d**), MCP-1 (**e,f**), cytochrome C (**g,h**), and IL-18 (**i,j**) was compared with percentage cortical injury in adenine-treated rats (left column, panels **a,c,e,g,i**) and in control rats (right column, panels **b,d,f,h,j**). Correlations at each cisplatin dose are shown in Supplementary Table S3. Values represent individual kidneys within each group.

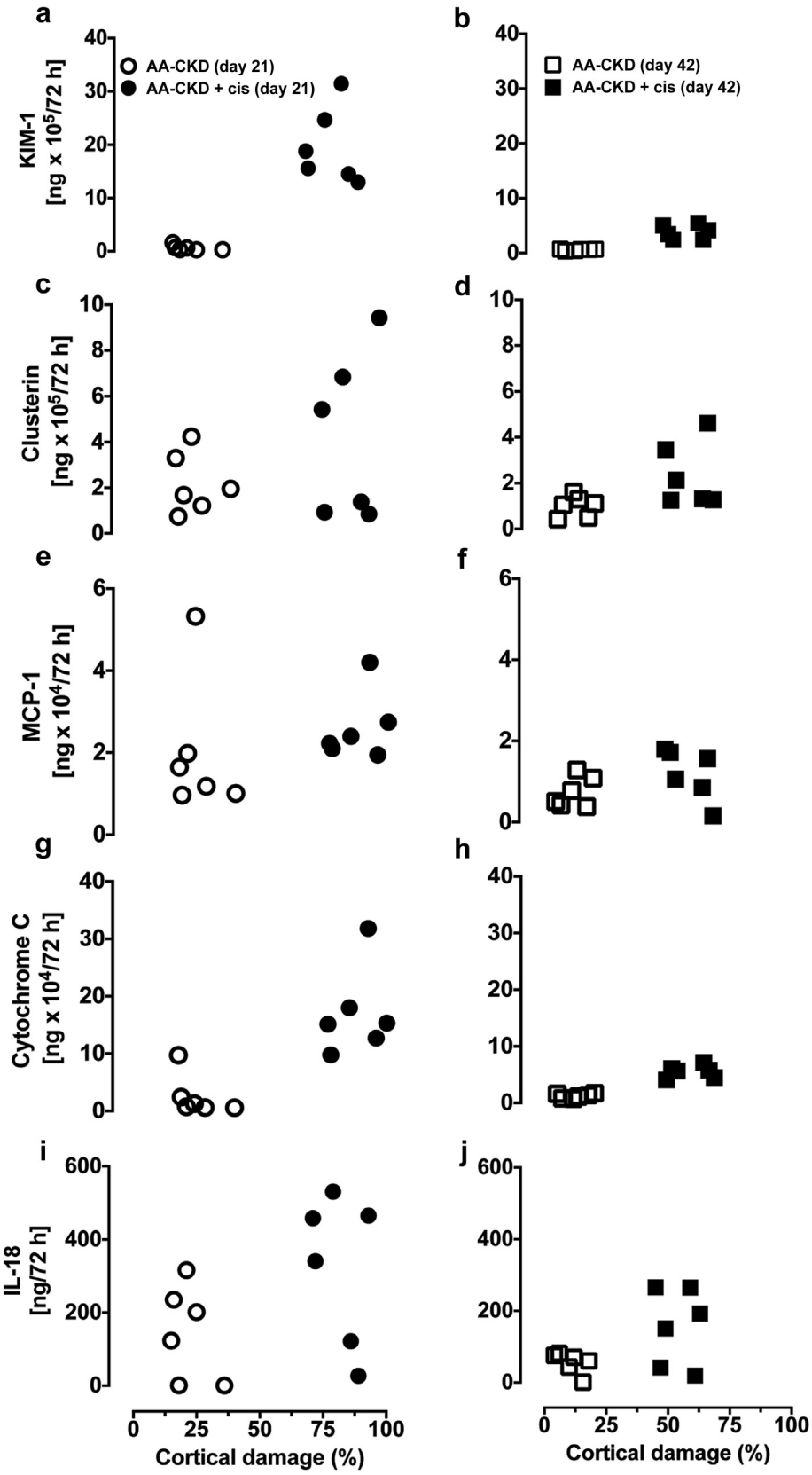

Figure 5 | Biomarker excretion versus cortical damage after acute kidney injury in aristolochic acid–induced chronic kidney disease (AA-CKD). Total biomarker excretion for 72 hours for KIM-1 (**a,b**), clusterin (**c,d**), MCP-1 (**e,f**), cytochrome C (**g,h**), and IL-18 (**i,j**) was compared with percentage cortical injury after cisplatin administration at day 21 (left column, panels **a,c,e,g,i**) and at day 42 (right column, panels **b,d,f,h,j**). Correlations at each cisplatin dose are shown in Supplementary Table S3. Values represent individual kidneys within each group.

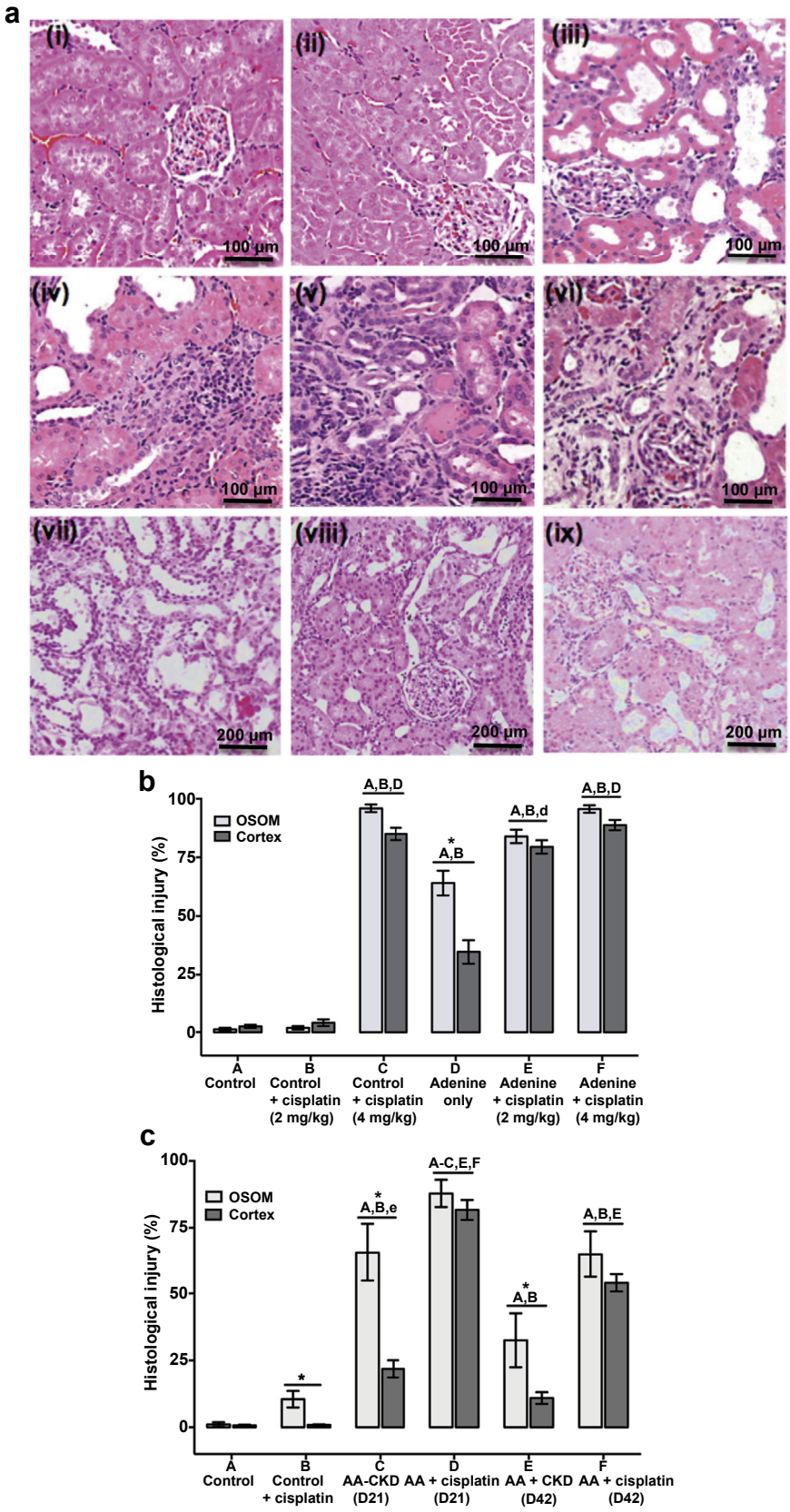

Figure 6 | Adenine- and aristolochic acid (AA)-induced chronic kidney disease (CKD) before and after induction of acute kidney injury. (**a**) Representative histology (hematoxylin and eosin staining; individual magnification levels indicated) of (i) control with normal histology; (ii) control + cisplatin (2 mg/kg): minimal tubulointerstitial damage with few apoptotic cells in the outer stripe of the outer medulla (OSOM); (iii) control + cisplatin (4 mg/kg): widespread tubulointerstitial damage in the OSOM and cortex; (iv) adenine only: injury (Continued)

respect to control kidneys. Consistent with urinary profiles, the 72-hour biomarker excretion was also reduced for all biomarkers after cis-induced AKI on day 42 versus day 21 in AA-induced CKD (Figure 5).

Renal histology

Histology is illustrated in Figure 6a, and regional differences are analyzed for adenine-induced CKD in Figure 6a and AA-induced CKD in Figure 6b. Adenine-induced CKD rats displayed normal histology on day 3. On day 63, controls displayed normal histology, and minimal histological injury was observed in control + cis (2 mg/kg) rats, and was limited to the OSOM (Figure 6). After 4 mg/kg cis, control rats displayed diffuse OSOM and cortical TID, characterized by tubular dilatation and apoptosis and infiltration with inflammatory cells (Figure 4). In the absence of cis, adenine-induced CKD kidneys at day 63 displayed diffuse injury to the OSOM, whereas less than half the cortex was injured with focal infiltration of inflammatory cells, patchy tubular dilatation, and apoptosis. After cis, there was widespread TID in both adenine + cis (2 mg/kg) and adenine + cis (4 mg/kg) groups, with features of both chronic and acute TID—namely tubular dilatation, proteinaceous cast formation, atrophy, apoptosis, and severe infiltration of inflammatory cells (Figure 4). In AA-induced CKD, histological damage was less at day 42 than at day 21 and comprised apoptosis in the OSOM and recruitment of tubulointerstitial inflammatory cells. After cis, TID in the OSOM and cortex, with apoptosis, tubular atrophy, dilatation, and infiltrating inflammatory cells, was pronounced after administration on day 21 versus day 42. As noted above, excretion of KIM-1 after cis was greater at day 21 (when there was more histological damage) than at day 42, and the degree of biomarker excretion correlated with the extent of injury (Figure 5 and Supplementary Table S3).

Apoptosis

Apoptosis was quantified by *in situ* labeling with ApopTag (EMD Millipore, Billerica, MA; Figure 7). There was positive labeling of tubular epithelium and interstitium in AA- and adenine-induced CKD and negligible labeling in controls. In both AA- and adenine-induced CKD, widespread apoptosis was limited to the OSOM, with focal areas in the cortex. After cis, apoptosis was diffuse in the OSOM and cortex irrespective of cis dose in adenine-induced CKD, and more severe after day 21 versus day 42 in AA-induced CKD ($P < 0.05$; Figure 5).

DISCUSSION

We previously demonstrated that AKI biomarker diagnostic performance is modified by reduced GFR.[9] It remains unknown how biomarker expression is altered by CKD or how to design a diagnostic panel to recognize and compensate for underlying CKD. Because patients may lose >50% GFR without an increase in sCr, subclinical CKD cannot be recognized without direct measurement of GFR or renal biopsy, which is rarely performed after recovery from AKI, particularly in the presence of a normal sCr level. To assess biomarker performance in analogous scenarios, we developed 2 nephrotoxin-induced models of subclinical CKD with >50% parenchymal renal injury but little or no increase in sCr. While the Star group studied the impact of CKD on sepsis and sepsis-induced AKI using a 5/6 nephrectomy model,[17] to our knowledge, this is the first study to examine AKI biomarkers of AKI in an intact kidney model with CKD. The results demonstrated that functional and damage biomarker profiles are significantly modified in diametrically different ways by prior CKD.

The adenine and AA models of CKD provide a useful tool profiling biomarkers of kidney damage after a second insult. Both models produced functional and structural changes that mimic human CKD, including, tubulointerstitial inflammation, fibrosis, and tubular atrophy.[12,15] After induction of CKD using either adenine or AA-1 followed by a period of recovery, animals were exposed to a second toxic insult, with cis, using either a low dose verified to produce no functional or parenchymal injury or a high dose causing AKI and elevated sCr level. As expected, because detection of nephrotoxic histological injury by urinary biomarkers is the basis[18] for qualification by the U.S. Food and Drug Administration as a valid damage biomarker,[19] most novel damage biomarkers ($_u$KIM-1, $_u$cytochrome C, $_u$MCP-1, $_u$clusterin, and $_u$IL-18) detected the induction of CKD, which is akin to a nephrotoxic AKI episode, without an increase in sCr despite extensive TID. However, $_u$IL-18 appeared important in the induction of CKD with adenine but not with AA. Adenine-induced CKD is characterized by early acute inflammatory response whereby IL-18, being a proinflammatory cytokine, is activated, followed by tubular epithelial cell apoptosis, tubulointerstitial inflammation, and fibrosis.[15] On the other hand, AA-induced CKD is characterized by early apoptosis presumably from DNA damage from specific AA–DNA adduct formation, ultimately leading to defective cell proliferation and development of tubular atrophy,[20] eventuating tubulointerstitial

Figure 6 | (Continued) predominantly in the OSOM; (v) adenine + cisplatin (2 mg/kg): injury limited to the OSOM; (vi) adenine + cisplatin (4 mg/kg): widespread OSOM and cortical injury with apoptosis and tubulointerstitial injury and inflammation; (vii) AA-induced CKD only (day 21): diffuse OSOM and ~25% cortical injury; (viii) AA-induced CKD only (day 42): segmental injury to the OSOM and minimal cortical injury; (ix) AA-induced CKD + cisplatin (day 42): severe diffuse injury to the OSOM and cortex. (**b,c**) Quantitation of regional injury. Data are expressed as mean ± SD; differences between OSOM and cortical injury indicated as * for $P < 0.025$. Statistical differences in cortical injury between groups is indicated using letters taken from the column headings, with uppercase letters indicating $P < 0.01$ and lowercase letters indicating $P < 0.05$.

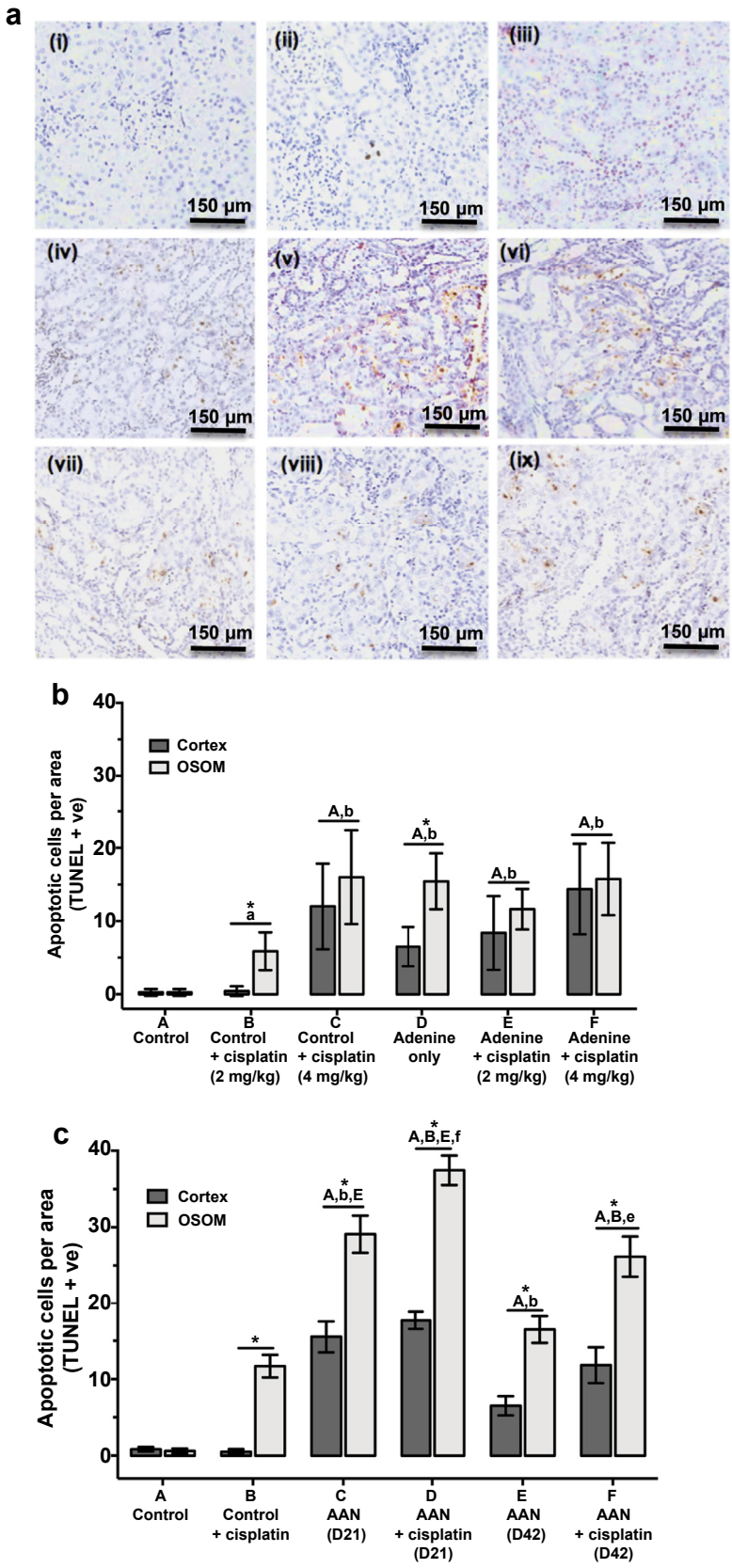

Figure 7 | Apoptosis in adenine and aristolochic acid (AA)-induced chronic kidney disease (CKD) before and after acute kidney injury. (**a**) Staining for ApopTag. (i) Control sections with negative staining; (ii) control + cisplatin (2 mg/kg): minimal and focal positive cells in the outer stripe of the outer medulla (OSOM) and cortex; (iii) control + cisplatin (4 mg/kg): widespread tubulointerstitial positive cells in the OSOM and cortex; (iv) adenine-only rats: staining predominantly in the OSOM and focal staining in the cortex; (Continued)

inflammation.[14] Nevertheless, after induction and time for recovery, all urinary biomarkers concentrations returned to baseline.

This induction phase illustrated that sCr concentration is normally an insensitive marker of mild to moderate acute injury in previously healthy rats. This is akin to in healthy humans,[21–23] in whom a large (≥25%) functional reserve may be anticipated, and consequently significant renal damage could occur without increasing sCr. However, these experiments illustrate that measurement of damage biomarkers following exposure to a toxin or a known renal insult would allow renal damage to be detected and perhaps even estimated noninvasively from total biomarker excretion.

Both functional and damage AKI biomarker profiles were modified by the presence of subclinical CKD. The sensitivity of sCr for detection of AKI was enhanced, as shown by the rapid increase after a low "subnephrotoxic" cis dose. Improvements in sCr performance are expected in CKD, because of the reciprocal relationship between GFR and sCr, so that logarithmic increases in sCr are seen for stepped decreases in GFR. Consistent with this, analysis of histological damage revealed that an increase in sCr did not occur until diffuse injury to the OSOM was coupled with at least 50% cortical damage.

In contrast, the increase in urinary damage biomarkers was delayed and diminished in the presence of subclinical CKD as hypothesized. While all biomarkers, with the exception of osteopontin, increased after subnephrotoxic cis in adenine-induced CKD, increases in $_u$KIM-1, $_u$MCP-1, and $_u$clusterin after high-dose cis were delayed for at least 1 day in subclinical adenine-induced CKD. These delays in damage biomarker production are consistent with our clinical studies of urinary KIM-1, NGAL, cystatin C, and GST in critically ill patients with CKD who developed AKI.[9]

There are several possible explanations for delayed and reduced urinary excretion of damage biomarkers in the presence of underlying CKD. Firstly, CKD may have reduced the number of viable nephrons,[24] reducing the maximum possible biomarker expression for a given severity of injury.[25] Secondly, alterations in the microvasculature associated with tubular injury and maladaptive repair[26] may have delayed or reduced urinary biomarker excretion. Thirdly, induction of CKD may activate repair or other protective mechanisms through preconditioning.[27] These studies were not designed to address these explanations. However, given the higher urinary damage biomarker concentrations observed when injury was induced early versus late in AA-induced CKD, and the long recovery (28 days) provided before cis in the adenine-induced CKD model, renoprotection by preconditioning appears very unlikely. In any case, these results suggest that biomarker thresholds should be lower or sampling delayed in the presence of CKD.

Biomarker performance in the presence of subclinical CKD was similar, regardless of whether CKD was induced by adenine or AA. However, the biomarker response was modified by the duration of recovery. Because induction of CKD with AA was rapid, the 2 durations of recovery in the AA-induced CKD model allowed exploration of the effect of different total background injury and acuity on biomarker responses. OSOM, cortical, and total injury before cis were less at day 42 than day 21 (Figure 7). After cis-induced AKI, the increment in cortical and OSOM injury was similar at both time points. Urinary concentrations of damage biomarkers after cis were greater at day 21 (than day 42) and also greater than in control kidneys. However, these relative increases were reversed when AKI was induced at day 42. Importantly, the temporal pattern of increase then resembled the more chronic pattern seen after 4 weeks' recovery in adenine-induced CKD, with delayed and reduced biomarker concentration compared with controls.

We speculate that the differences in pattern at day 21 versus day 42 reflect more unrecovered or ongoing acute injury at day 21. This is supported by the greater degree of apoptosis in both the OSOM and cortex at day 21 (Figure 7) and by the reduction in cortical damage at day 42 (Figure 6). Thus, at day 21, acute injury from cis was superimposed on ongoing or residual acute injury from AA. After more prolonged recovery at day 42, the biomarker response resembled that seen in the adenine-induced CKD model. Changes in biomarker excretion were consistent with the changes in urinary concentration and correlated with the degree of background histological damage.

After the initial observations by McIlroy[10] and ourselves,[9] the few clinical studies that have looked at the interaction between biomarker performance and underlying CKD showed modest or no interaction. TIMP2 x IGFBP7 performance was unimpaired by the presence of CKD in a recent retrospective analysis.[28] However, because the cutoffs used to predict progression from stage 1 AKI were derived using very heterogeneous populations, this may have accounted for any heterogeneity imposed by preoperative CKD. Certainly, diagnosis of AKI has not been uniformly robust with this biomarker combination, and context and timing remain important.[29]

Post hoc analysis of the TRIBE data showed that in patients developing AKI after cardiac surgery, higher postoperative

Figure 7 | (Continued) (v) adenine + cisplatin (2 mg/kg) and (vi) adenine + cisplatin (4 mg/kg) exhibited widespread staining in the OSOM and cortex; (vii) AA-induced CKD only (day 21): segmental staining in the OSOM with focal areas in the cortex; (viii) AA-induced CKD only (day 42): segmental staining in the OSOM with minimal staining in the cortex; (ix) AA-induced CKD + cisplatin (day 21): diffuse staining in the OSOM and cortex. (**b,c**) Quantitation of regional apoptosis. Data are expressed as mean ± SD; differences between OSOM and cortical apoptosis indicated as * for $P < 0.05$. Statistical differences in cortical injury between groups are indicated using letters taken from the column headings, with uppercase letter indicating $P < 0.01$ and lowercase letters indicating $P < 0.05$.

urinary IL-18, NGAL, KIM-1, L-FABP, cystatin C, and albumin levels were recorded for those patients with eGFR > 60 ml/min/1.73 m^2 and lower values with preoperative CKD. However, analysis of the log-transformed data showed only a modest interaction of AKI with baseline eGFR for log-transformed early postoperative data (for IL-18 and L-FABP) values and CKD (baseline eGFR < 60 ml/min/1.73 m^2).[30] Differences in study populations, and diagnosis using quintile ratios rather than specific cutoffs, may explain some of differences in these outcomes. However, the Koyner post–cardiac surgery data suggest that the impact of preoperative CKD on biomarker performance was likely to be small after cardiac surgery, even in those biomarkers for which CKD impacted diagnostic performance (i.e., for urinary IL-18, L-FABP, and cystatin C). These few studies suggest that more data are needed to understand the impact of CKD on biomarker diagnostic performance in the clinical arena.

Overall, the results suggest that identification of AKI is not more difficult in the presence of CKD because markers of function such as sCr are more sensitive. However, the results highlight the low sensitivity of sCr during induction of CKD, because this structural injury was only identified by damage biomarkers and not sCr. The results confirm the ubiquitous clinical experience that surrogate markers of GFR such as sCr are inadequate for the noninvasive identification of subclinical CKD. They suggest this phase of CKD requires direct measurement of GFR for detection. However, we speculate that these changes may be too subtle even with direct measurement of GFR unless a perturbation of renal function (analogous to a stress test) is also applied. A standardized measurement of renal reserve might serve as such a stress test.

In conclusion, urinary levels of $_u$KIM-1, cytochrome c, clusterin, $_u$MCP-1, and $_u$IL-18 were sensitive early detectors of kidney damage compared with sCr during induction of both adenine- and AA-induced CKD. Once structural CKD injury was established, both function marker and damage biomarkers sensitively detected AKI. However, the sensitivity of sCr was enhanced while the increase in urinary damage biomarkers was delayed and diminished. These results suggest that AKI biomarker thresholds should be lower or sampling delayed in the presence of CKD. Both adenine- and AA-induced CKD models may facilitate further exploration of subclinical CKD.

METHODS

Sprague-Dawley rats aged 6 to 8 weeks were maintained at the Biological Resource Centre at the University of New South Wales. Rats were given *ad libitum* access to standard or adenine-supplemented rodent chow (Harlan Laboratories, Madison, WI) and tap water throughout, weighed daily, and monitored for physical distress. All animal work was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013) and approved by the animal ethics committee at the University of New South Wales (protocol ID 13/74A and 12/38A).

Adenine-induced CKD

Forty-eight rats were randomly allocated to 6 groups: (i) adenine only ($N = 8$), (ii) adenine + cis (2 mg/kg) ($N = 8$), (iii) adenine + cis (4 mg/kg) ($N = 8$), (iv) control + cis (2 mg/kg) ($N = 8$), (v) control + cis (4 mg/kg) ($N = 8$), and (vi) control + saline ($N = 8$). CKD was induced by feeding a 0.025% adenine-supplemented diet (Sigma-Aldrich, Castle Hill, Australia) for 28 days, after which standard chow was fed until day 56. Control animals received standard chow. On day 56, rats were administered either a nephrotoxic (4 mg/kg) or subnephrotoxic (2 mg/kg) i.p. cis dose. Determined from dose response of sCr and renal histology, a cis dose given to a normal rat fed standard chow causing no change to sCr or histology was deemed subnephrotoxic. In control animals, saline was administered instead of cis. Blood and urine samples were collected on days 0, 1, 3, 7, 14, 21, 28, 42, 56, 57, 58, 59, and 63. Blood was collected from saphenous vein and urine samples obtained by overnight metabolic caging (16 hours); urine was collected at 4°C. On day 63, rats were killed (i.p. pentobarbital sodium) followed by removal of kidneys for histology.

AA-induced CKD

The active ingredient AA-1 (Sigma-Aldrich) was injected daily (10 mg/kg i.p.) for 5 days. Animals were monitored daily until day 21 ($n = 6$) or 42 ($n = 6$), followed by cis i.p. at a nephrotoxic 4 mg/kg ($n = 6$) or subnephrotoxic 2 mg/kg ($n = 6$) dose. Animals were then monitored to days 28 and 49, respectively.

Biomarker analysis

Serum and urine creatinine were measured enzymatically using Konelab 630 automated analyzer (Thermo Fisher, Waltham, MA). $_u$Cytochrome C was measured in duplicate by enzyme-linked immunosorbant assay (R&D Systems, Minneapolis, MN) as per the manufacturer. Intra- and inter-assay variability was < 10%. $_u$KIM-1, $_u$clusterin, $_u$IL-18, $_u$MCP-1, and osteopontin were measured using Bioplex panel 1 (Bio-Rad, Gladesville, Australia). Urinary biomarkers were normalized to urinary creatinine concentration. Total biomarker excreted in 72 hours after AKI was calculated from daily urine volume and absolute biomarker concentration.

Histopathology

Midcoronal slices (5 mm) were immersion-fixed in 10% formalin at room temperature. Sections fixed in 10% formalin were dehydrated overnight in graded alcohol (Tissue-Tek VIP 4 Tissue processor; Sakura, Olympus, Australia) and embedded in paraffin. Apoptosis was quantified with ApopTag *in situ* detection kit (Merck Group, Kilsyth, Australia) and with morphology.[15] Both ApopTag and hematoxylin and eosin–stained sections were examined using Aperio slide scanner and Image Scope Analysis Software (Aperio, Vista, CA) at x200 magnification) for TID and ApopTag-positive cells by 1 observer (LS) blinded to treatment. TID was scored (0 = no histological injury; 1 = <25%; 2 = 26% to 50%; 3 = 51% to 75%; and 4 = 76% to 100%) per section and imaged by Aperio slide scanner at ×200 to quantify cortical and OSOM of ApopTag-positive cells.

Statistical analysis

Biomarker concentrations were analyzed by using repeated measures 2-way analysis of variance followed by Dunn's *post hoc* analyses adjusted for multiple comparisons. Unpaired *t*-tests determined significance between groups at identical time points: $^*P < 0.05$ from controls and $^{\#}P < 0.05$ from baseline. Biomarker excretion was correlated with histological cortical damage (%) using Pearsons r^2.

All statistical analyses used GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Normalized biomarker concentrations after cis in adenine-induced chronic kidney disease.

Table S2. Normalized biomarker concentrations after cis in aristolochic acid–induced chronic kidney disease.

Table S3. Biomarker excretion versus cortical injury.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES

1. Collaborators. GBoDS. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. 2015;386:743–800.
2. Chadban SJ, Briganti EM, Kerr PG, et al. Prevalence of kidney damage in Australian adults: The AusDiab kidney study. *J Am Soc Nephrol*. 2003;14: S131–S138.
3. Matsushita K, Coresh J, Sang Y, et al. Estimated glomerular filtration rate and albuminuria for prediction of cardiovascular outcomes: a collaborative meta-analysis of individual participant data. *Lancet Diabetes Endocrinol*. 2015;3:514–525.
4. Chawla LS, Eggers PW, Star RA, Kimmel PL. Acute kidney injury and chronic kidney disease as interconnected syndromes. *N Engl J Med*. 2014;371:58–66.
5. Coca SG, Yusuf B, Shlipak MG, et al. Long-term risk of mortality and other adverse outcomes after acute kidney injury: a systematic review and meta-analysis. *Am J Kidney Dis*. 2009;53:961–973.
6. Chawla LS, Amdur RL, Amodeo S, et al. The severity of acute kidney injury predicts progression to chronic kidney disease. *Kidney Int*. 2011;79: 1361–1369.
7. Bohle A. Change of paradigms in nephrology–a view back and a look forward. *Nephrol Dial Transplant*. 1998;13:556–563.
8. Aasarod K, Bostad L, Hammerstrom J, et al. Renal histopathology and clinical course in 94 patients with Wegener's granulomatosis. *Nephrol Dial Transplant*. 2001;16:953–960.
9. Endre ZH, Pickering JW, Walker RJ, et al. Improved performance of urinary biomarkers of acute kidney injury in the critically ill by stratification for injury duration and baseline renal function. *Kidney Int*. 2011;79:1119–1130.
10. McIlroy DR, Wagener G, Lee HT. Neutrophil gelatinase-associated lipocalin and acute kidney injury after cardiac surgery: the effect of baseline renal function on diagnostic performance. *Clin J Am Soc Nephrol*. 2010;5:211–219.
11. Pianta TJ, Succar L, Davidson T, et al. Monitoring treatment of acute kidney injury with damage biomarkers [e-pub ahead of print]. *Toxicol Lett*. http://dx.doi.org/10.1016/j.toxlet.2017.01.001.
12. Fong D, Ullah MM, Lal JG, et al. Renal cellular hypoxia in adenine-induced chronic kidney disease. *Clin Exp Pharmacol Physiol*. 2016;43:896–905.
13. Pozdzik AA, Salmon IJ, Debelle FD, et al. Aristolochic acid induces proximal tubule apoptosis and epithelial to mesenchymal transformation. *Kidney Int*. 2008;73:595–607.
14. Pozdzik AA, Salmon IJ, Husson CP, et al. Patterns of interstitial inflammation during the evolution of renal injury in experimental aristolochic acid nephropathy. *Nephrol Dial Transplant*. 2008;23:2480–2491.
15. Diwan V, Mistry A, Gobe G, Brown L. Adenine-induced chronic kidney and cardiovascular damage in rats. *J Pharmacol Toxicol Methods*. 2013;68: 197–207.
16. Fountain KJ, Kloss A, Garibyan I, et al. Analysis of creatinine in mouse and rat serum by ion exchange high performance liquid chromatography for in vivo studies of renal function. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;846:245–251.
17. Leelahavanichkul A, Huang Y, Hu X, et al. Chronic kidney disease worsens sepsis and sepsis-induced acute kidney injury by releasing High Mobility Group Box Protein-1. *Kidney Int*. 2011;80:1198–1211.
18. Waring WS, Moonie A. Earlier recognition of nephrotoxicity using novel biomarkers of acute kidney injury. *Clin Toxicol (Phila)*. 2011;49: 720–728.
19. Dieterle F, Sistare F, Goodsaid F, et al. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. *Nat Biotechnol*. 2010;28:455–462.
20. Lebeau C, Debelle FD, Arlt VM, et al. Early proximal tubule injury in experimental aristolochic acid nephropathy: functional and histological studies. *Nephrol Dial Transplant*. 2005;20:2321–2332.
21. Barai S, Gambhir S, Prasad N, et al. Functional renal reserve capacity in different stages of chronic kidney disease. *Nephrology*. 2010;15: 350–353.
22. Sharma A, Zaragoza JJ, Villa G, et al. Optimizing a kidney stress test to evaluate renal functional reserve. *Clin Nephrol*. 2016;86:18–26.
23. Ronco C, Rosner MH. Acute kidney injury and residual renal function. *Crit Care*. 2012;16:144.
24. Kriz W, LeHir M. Pathways to nephron loss starting from glomerular diseases-insights from animal models. *Kidney Int*. 2005;67:404–419.
25. Pickering JW, Endre ZH. Acute kidney injury urinary biomarker time-courses. *PloS One*. 2014;9:e101288.
26. Basile DP, Bonventre JV, Mehta R, et al. Progression after AKI: Understanding Maladaptive Repair Processes to Predict and Identify Therapeutic Treatments. *J Am Soc Nephrol*. 2016;27:687–697.
27. Endre ZH. Renal ischemic preconditioning: finally some good news for prevention of acute kidney injury. *Kidney Int*. 2011;80:796–798.
28. Heung M, Ortega LM, Chawla LS, et al. Common chronic conditions do not affect performance of cell cycle arrest biomarkers for risk stratification of acute kidney injury. *Nephrol Dial Transplant*. 2016;31: 1633–1640.
29. Vijayan A FS, Askenazi DJ, Cerda J, et al.; American Society of Nephrology Acute Kidney Injury Advisory Group. Clinical use of the urine biomarker [timp-2] × [igfbp7] for acute kidney injury risk assessment. *Am J Kidney Dis*. 2016;68:19–28.
30. Koyner JL, Coca SG, Thiessen-Philbrook H, et al. Urine biomarkers and perioperative acute kidney injury: the impact of preoperative estimated GFR. *Am J Kidney Dis*. 2015;66:1006–1014.