Comparison of serum creatinine and serum cystatin C as biomarkers to detect sepsis-induced acute kidney injury and to predict mortality in CD-1 mice

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Seum creatinine (SCr) is currently used to detect and stage acute kidney injury (AKI) and chronic kidney injury despite well-known limitations. Creatine is synthesized primarily in the liver and then transported to skeletal muscle for use in storing ATP (3). Creatinine (molecular mass: 113 Da), a metabolic end product of creatine, is released into plasma, freely filtered through the glomerulus, also secreted but not absorbed by renal tubules. Whereas SCr is predominantly determined by glomerular filtration rate (GFR), SCr is also influenced by nonrenal factors that alter creatinine generation (muscle mass, dietary creatine intake, and liver function) and elimination (gastrointestinal excretion) (3). We previously demonstrated that creatinine production is reduced in sepsis, which limits the ability of SCr to detect and stage sepsis-induced AKI (sepsis-AKI) (5). An endogenous molecule with different properties could overcome these limitations, such as serum cystatin C (sCysC; molecular mass: 13.3 kDa) (1) a proteinase inhibitor that J prevents connective tissue destruction (24), (2) is constantly produced by most nucleated cells in the body, (3) is freely filtered by the glomerulus, and (4) is then entirely reabsorbed and catabolized in the proximal tubule (28, 31). sCysC has been proposed by some as a more ideal endogenous biomarker of chronic kidney function (4, 12, 21, 28), although it is also affected by age, sex, muscle mass, smoking, thyroid function, and malignancies (2).

Whether sCysC is a better biomarker of kidney injury is a matter of controversy, and it may depend on the context of use (14, 15). Within the context of chronic kidney disease, a combined creatinine-CysC estimated GFR (eGFR) equation can perform somewhat better than equations based on either biomarker alone (15).

In the setting of human AKI, urinary CysC predicts AKI 48–72 h before SCr, whereas sCysC may detect AKI 1–2 days earlier than SCr in intensive care unit patients who developed AKI and were classified according to RIFLE criteria (13). sCysC also outperforms SCr as an early biomarker of AKI in the emergency room setting (29) and after cardiopulmonary bypass in children (17).

But while some studies have shown the superiority of sCysC as an early biomarker of AKI, other studies have shown that sCysC performs as well as, or even worse, than SCr. In a study by Wald et al. (32), serial measurements of sCysC in adult patients undergoing cardiopulmonary bypass correlated with the development of AKI, but the discriminatory capacity of sCysC as an early biomarker of AKI was very limited. In the postoperative period after cardiac surgery in elderly patients, sCysC and SCr detected AKI similarly (25), and in a multicenter prospective observational cohort study involving a heterogeneous adult population admitted to an intensive care unit, both urinary CysC and sCysC were poor early predictors of AKI and the need for renal replacement therapy (26).

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For sCysC to be considered a better biomarker of AKI than SCr in some settings but not in others, rational criteria are needed for each setting (context of use). Early detection biomarkers are especially difficult to establish in patients, as the timing of the initial renal injury is often difficult to discern. Therefore, we compared the ability of SCr and sCysC to detect kidney injury caused by sepsis (sepsis-AKI) under more controlled circumstances using an experimental model of sepsis in mice.

METHODS

Animals and animal models. We followed National Institutes of Health (NIH) criteria for the use and treatment of laboratory animals. All experiments were conducted on 6- to 8-wk-old male CD-1 mice (Charles River Laboratories) except that 12- to 16-wk-old male CD-1 mice were used for blood collections at 6 h after the induction of sepsis and subsequent survival analysis. All animals had free access to water and chow and were monitored postoperatively for symptoms including piloerection, spontaneous activity, response to stimuli, labored breathing, and eye grooming. All procedures were performed under isoflurane anesthesia (including the euthanasia of morbidly ill mice, per protocol).

Cecal ligation and puncture (CLP) was performed to induce sepsis as previously described (18, 20, 33). In brief, the cecum was ligated 12 mm from its tip and then punctured twice with a 21-gauge needle. Bilateral nephrectomy (BiNx) was performed as previously described (5); briefly, both kidneys were decapsulated to avoid adrenal damage and then removed via flank incisions. BiNx was performed at the same time as CLP in the BiNx + CLP group. For sham surgery, the cecum and/or kidneys were identified via simultaneous incisions. Normal saline (15 ml/kg) was given intraperitoneally immediately after all surgeries and then either antibiotic (imipenem-cilastatin, 14 mg/kg in 1 ml normal saline) for CLP or normal saline for BiNx was given subcutaneously at 6 h. All mice subjected to any surgical procedure also received buprenorphine solution (0.05 mg/kg ip) immediately after and at 6 h after surgery. When we measured GFR 6 h after CLP, we delayed fluid administration so that it would not dilute inulin before blood sampling.

GFR measurements in conscious mice. GFR was measured at 0, 3, 6, 12, or 18 h after CLP by FITC-labeled inulin clearance (7). Each mouse was studied only once at a single time point (n = 5–6 mice/time point). One microcapillary tube of blood (50 μl) was collected via a tail vein for the measurement of SCr, blood urea nitrogen (BUN), and sCysC at the specified time point post-CLP. A single dose of FITC-inulin (3.74 μl/g body wt) was injected into the retroorbital plexus, and 5-μl blood samples were collected from the tail vein 10, 15, 35, 55, and 75 min afterward. Plasma fluorescence was measured by a Nanodrop-ND-3300 fluorescence spectrometer (Nanodrop Technologies, Wilmington, DE). GFR was then calculated using a two-compartment model, as previously described (7). The correlation between SCr, sCysC, and BUN at the specified time post-CLP and inulin GFR (iGFR) in the individual mice was calculated (SigmaStat 3.1, Systat Software, Point Richmond, CA).

Blood chemistries. Fifty microliters of blood were collected by the retroorbital approach before surgery (0 h) and at 3, 6, 12, and 18 h after surgery (n = 5–6 mice/time point), and body weight was recorded. SCr was measured by HPLC (34), BUN by colorimetric assay (QuantiChrom Urea assay kit DIUR-500, Hayward, CA), and mouse sCysC by ELISA (BioVendor, Candler, NC). Under anesthesia at 18 h after surgery, blood was collected after cardiac puncture, and mice were euthanized. Serum aspartate transaminase, alanine transaminase, and lactate dehydrogenase were measured by an autoanalyzer (Hitachi 917, Boehringer Mannheim, Indianapolis, IN), and serum TNF-α, IL-6, and IL-10 were measured by ELISA (R&D Systems, Minneapolis, MN).

Survival experiments. Six hours after the induction of CLP-induced sepsis, 50 μl of blood were collected by the retroorbital sinus approach under avertin anesthesia. Blood collection from younger (6–8 wk old) mice after sepsis resulted in rapid mortality (data not shown); hence, the use of older (12–16 wk old) mice for survival experiments. Animals were monitored for survival every 4–8 h after surgery, and the time to death was recorded for each animal. SCr, sCysC, and BUN were measured as described above. Mice were given fluids and buprenorphine immediately after CLP; fluids, antibiotic, and buprenorphine were given starting at 6 h after sepsis and then given every 12 h to the time of death. Morbidly ill mice were euthanized per protocol.

Production and kinetics of sCysC. Production and kinetics of sCysC were performed in BiNx and BiNx + CLP groups only. We took advantage of the otherwise stable sCysC level 12 h after BiNx or BiNx + CLP to measure the pharmacokinetics of injected recombinant CysC. At 12 h after BiNx or BiNx + CLP, 25 μl of capillary blood were collected via the retroorbital sinus, and recombinant CysC...
Fig. 2. Time course of kidney dysfunction during sepsis-induced acute kidney injury (AKI) and cross correlation with renal function biomarkers. After sepsis-induced AKI, sCysC (A; $R^2 = 0.924$), SCr (B; $R^2 = 0.743$), and BUN (C; $R^2 = 0.813$) all increased, and their reciprocals were correlated with iGFR, which decreased. A linear regression analysis of the relationship between each biomarker’s reciprocal and iGFR was performed. iGFR and estimated GFR (eGFR) values based on sCysC (E), SCr (F), and BUN (G) values were plotted against each other. A multiple regression model was created combining SCr and sCysC ($R^2 = 0.944$). The relationship between a weighted composite value of SCr and sCysC generated based on the coefficients of the multiple regression model is shown (D), together with iGFR plotted against composite eGFR (H).
distribution ($V_d$) was calculated as follows: $V_d = \frac{\text{injected dose}}{[s\text{CysC}_{\text{peak postinjection}} - 12 \text{ h} + 5 \text{ min}] - s\text{CysC}_{\text{preinjection}} - 12 \text{ h}}$, and the sCysC net production was calculated as follows: net production = ($s\text{CysC}_{\text{12 h}} - s\text{CysC}_{\text{0 h}}$) × $V_d$, as nonrenal clearance cannot be distinguished from production. An alternative noncompartmental method of calculating $V_d$ (Phoenix WinNonlin software, version 6.02, Pharsight, Mountain View, CA) yielded similar results for $V_d$ estimates.

Statistical analysis. All data are expressed as means ± SE. ANOVA with Bonferroni’s multiple comparison correction was performed using Prism 4.0 (Graphpad Software). We compared survival curves using a log-rank test (Prism 4.0, Graphpad Software). The area under the receiver-operating characteristic curve was calculated for

![Fig. 3](image)

Fig. 3. Time course of sCysC, SCr, and BUN after CLP-induced sepsis alone or after bilateral nephrectomy (BiNx). A–C: time courses of sCysC (A), SCr (B), and BUN (C) in CLP and BiNx ($n = 5–6$ mice/group). Two-way ANOVA was performed with Bonferroni post hoc analysis. *$P < 0.05$, **$P < 0.01$, and #$P < 0.001$, BiNx vs. CLP at each time point.

(5 μg, R&D Systems) was injected by the tail vein. Additional samples were taken at 5, 15, 30, 60, 120, and 180 min to determine peak levels, and these limited time points enabled us to qualitatively confirm a single compartment with no substantial distributive phase. sCysC levels after CysC administration at 12 h were adjusted for an extrapolated production rate from 0 to 12 h. The volume of

![Fig. 4](image)

Fig. 4. A–C: time courses of sCysC (A), SCr (B), and BUN (C) in BiNx with and without CLP ($n = 5–6$ mice/group). Two-way ANOVA was performed with Bonferroni post hoc analysis. *$P < 0.05$ and #$P < 0.001$, CLP vs. BiNx + CLP at each time point. ns, Not significant.
each biomarker for each time point during the survival study (Prism 4.0, Graphpad Software). We used the Statsmodels software package (python programming language, http://statsmodels.sourceforge.net/) to perform a linear regression analysis on the reciprocal value of the biomarkers. A weighted regression analysis was used to validate results, with weights based on inverse variances, when visual inspection of residual plots indicated a possible deviation from homoscedasticity. Models were compared using the Akaike information criterion. *P values of <0.05 were accepted as statistically significant.

RESULTS

sCysC outperforms SCr and BUN as a renal function biomarker early in the course of sepsis-AKI. We simultaneously measured SCr, BUN, sCysC, and FITC-iGFR in conscious mice at 0, 3, 6, 12, and 18 h after CLP using 5–6 mice/time point. At 3 h after CLP, sCysC was increased threefold over baseline (0 h) sCysC. At this time point, both SCr and BUN less than doubled their values over baseline, and the sCysC increase over baseline was significantly higher than increases in SCr and BUN (Fig. 1A). At 12 and 18 h, sCysC also had significantly higher increases over baseline compared with SCr and BUN, whereas at 6 h, the increased biomarker levels were not statistically different (Fig. 1A). At 3 h after CLP, GFR dramatically decreased >50% (Fig. 1B); this was associated with a 3.2-fold increase in sCysC and a 1.7-fold increase in BUN but no change in SCr (1.16-fold; Fig. 1A). Accordingly, both sCysC and BUN outperformed SCr as early biomarkers of sepsis-AKI, with sCysC being the best early biomarker. At 18 h after sepsis, GFR was reduced by >90% (from 428 to 5 µl/min; Fig. 1B), which was associated with 12.7-, 4.8-, and 8.2-fold increases in sCysC, BUN, and SCr, respectively (Fig. 1A). In addition to detecting changes in GFR early in the progression of disease, accurate assessment of GFR is also important. The level of each biomarker was correlated to iGFR by including all time points. Because there is a reciprocal relationship between GFR and net accumulation of each circulating biomarker, we used 1/biomarker values to avoid an...
Sepsis blunts BiNx-induced increases in sCysC and SCr. We (5) have previously demonstrated that sepsis decreases creatinine production. To determine if sepsis also affects sCysC kinetics, CLP was performed simultaneously with BiNx. Loss of kidney function by BiNx increased sCysC, SCr, and BUN, as expected (Fig. 4, A–C); BiNx combined with sepsis blunted these increases in sCysC at 18 h and SCr at 12 and 18 h but did not blunt BUN increases (Fig. 4, A–C). In contrast, BiNx combined with sepsis significantly increased other inflammatory and organ damage parameters (inflammatory cytokines, aspartate transaminase, alanine transaminase, and lactate dehydrogenase) compared with BiNx alone (Fig. 5). Hence, consistent with our previous study with SCr (5), we now demonstrate that within the context of BiNx, sCysC also decreases after sepsis, in contrast to other circulating biomarkers, which do not decrease.

Sepsis decreases sCysC production and may increase non-renal sCysC elimination after BiNx without changing Vd. We next determined whether changes in sCysC kinetics (i.e., increased Vd; production, metabolism, or elimination) could account for the reduction of sCysC after sepsis. Exogenous recombinant CysC was injected, and blood was sampled frequently (see METHODS). Vd of sCysC was not different between BiNx + sham and BiNx + CLP groups (Fig. 6A), similar to our previous data on the FITC-inulin space (5). Additionally, there were no differences in body weights between BiNx + sham and BiNx + CLP groups (data not shown), as previously reported (5). Next, we found that sepsis reduced estimated net sCysC production (Fig. 6B); from this, we can infer that it may have increased nonrenal elimination (see METHODS) (Fig. 6C).

Serum CysC does not outperform SCr on predicting mortality in the setting of sepsis. SCr, sCysC, and BUN were measured at 6 h after CLP surgery, and the time to death was measured. Post hoc assignment of mice to low and high biomarker groups, using the median as a threshold, demonstrated statistically significant differences in the Kaplan-Meier survival curves for SCr, sCysC, and BUN (Fig. 7A). All mice died within 52 h. SCr, sCysC, and BUN were each able to predict the time to death (Fig. 7B), and higher biomarker median values were associated with decreased survival (Fig. 7A). Both SCr (P < 0.001) and sCysC (P < 0.001) were superior to BUN, and SCr was stronger than sCysC (P = 0.015) in predicting the time to death. Combining multiple biomarkers in the analysis did not improve on SCr alone. In contrast, there was no difference in the ability of SCr and sCysC to classify animals to earlier and later mortality groups. Receiver-operating characteristic curves constructed compar...

Fig. 6. Effect of sepsis on CysC volume of distribution (Vd), production, and elimination. A: Vd of CysC was measured at 5 min (as baseline) and at 12 h after surgery. B: estimated CysC production was measured at 0–12 h after CLP surgery (n = 5–6 mice/group). C: clearance was measured at 12 h after CLP surgery (n = 5–6 mice/group). *P < 0.05 vs. baseline.
AUC Serum CysC vs. AUC Serum Creatinine at different time points

\[ p = \text{ns} \]
ing mortality at earlier and later time points demonstrated no difference between SCr and sCysC (Fig. 7C).

DISCUSSION

An ideal serum kidney filtration biomarker should be constantly produced, freely filtered, neither secreted nor reabsorbed by the renal tubules, and lack nonrenal elimination pathways (27). The biomarker might either have fast kinetics to rapidly track minute changes in GFR or slower kinetics that integrate changes in GFR over a long time interval (similar to glucose vs. HbA1C). Unfortunately, the production rate of creatinine is influenced by many extrarenal factors (muscle mass, age, sex, and reduced production in sepsis-AKI) and tubular secretion (3). Similarly, BUN is influenced by nonconstant production and significant tubular reabsorption, especially during volume depletion (6). In contrast, sCysC production is more predictable, although it is modestly influenced by smoking, obesity, hyperthyroidism, and low-grade chronic inflammation (10, 11, 16), but it is largely unaffected by acute inflammation, including sepsis-induced inflammation (19, 23, 28). Thus, sCysC could meet most of the ideal filtration biomarker criteria with less interindividual variation (9).

At least two classes of AKI biomarkers are needed, each with a different context of use or role in clinical decision making: an early detection biomarker that is tightly coupled to GFR, enabling rapid detection after the insult with an opportunity to identify patients that may be at a higher risk, and a prognosis biomarker that can predict severity and/or mortality.

We demonstrated in an animal model of sepsis that sCysC changes rapidly after injury and that a sCysC-based eGFR equation better reflects iGFR throughout the course of sepsis-AKI. In comparison, BUN increased rapidly, but a BUN-based eGFR equation only moderately correlated with iGFR, SCr increased slowly, and a SCr-based eGFR equation only poorly reflected GFR. The combination of sCysC with SCr, but not BUN, further improved eGFR equations when correlated to iGFR. However, sCysC is still not an ideal biomarker because sepsis decreases the production and potentially increases the nonrenal clearance of sCysC. Furthermore, sCysC did not outperform the widely used SCr to predict mortality. The combination of SCr with sCysC also did not improve the performance of either individual biomarker with respect to sepsis mortality.

**sCysC outperforms creatinine early after AKI.** GFR cannot be calculated in patients from SCr during AKI until a steady state is reached days after injury; this period is prolonged because of the slow creatinine kinetics and decreased production (8). Therefore, we measured iGFR by the best available method of plasma disappearance of FITC-inulin, which uses a two-compartment model of two-phase compartment decay, to approximate how quickly GFR falls during sepsis. This iGFR measurement has limitations during sepsis because 1) the calculation is based on an assumption that GFR does not change appreciably during the 75-min sampling window and 2) the number of samples that can be collected is limited by potential artifacts introduced by multiple blood draws. A more sophisticated model could be developed, but there are limits to how many time points can be collected before the collection itself can cause its own artifacts. Nevertheless, sepsis rapidly reduced iGFR by >50% as early as 3 h after CLP (Fig. 1B), even before systemic sepsis symptoms, such as lethargy, diminished response to stimulus, or piloerection, developed at 6 h. Despite these large and rapid changes in iGFR, SCr was slow to react; sCysC and BUN had much faster kinetics; both increased rapidly and achieved a steady state approximately within 12 h (Fig. 1A).

**Influence of sepsis on sCysC production and metabolism.** We have recently found that sepsis blunted the increase in SCr after sepsis in BiNx mice and that creatinine production was reduced by sepsis (5). In the present study, we found that sepsis similarly blunted the increase in sCysC after sepsis in BiNx mice.

The effect of sepsis on sCysC was complicated, as sepsis both reduced sCysC production and may have also enhanced nonrenal clearance. The latter could be due to increased clearance by the reticuloendothelial system (31). Both of these could conspire to reduce the ability of sCysC to function as an early detection biomarker, even though sCysC did increase earlier (at 3 h) than SCr and BUN in the same mice. Although BUN did not correspond to iGFR as well as sCysC, it also increased rapidly after BiNx or CLP, and CLP did not blunt the increase observed after BiNx. As such, although BUN is not the best biomarker for GFR, it may still be useful for detecting early changes in GFR. The usefulness of this early rise may be somewhat diminished by 6 h when systemic symptoms of sepsis begin to manifest, as both sCysC and SCsC outperformed BUN as predictors of mortality.

**AKI biomarkers and mortality.** In this study, although sCysC was increased more than SCr early after CLP, it did not outperform SCr in predicting mortality. The areas under the receiver-operating characteristic curves of sCysC and SCr with the outcome of mortality were comparable. Our result is consistent with previous work by Perianayagam et al. (22), which demonstrated that a single measurement of sCysC at the time of nephrology consultation did not surpass SCr on predicting in-hospital mortality among critically ill patients diagnosed with AKI. To date, very few clinical studies have analyzed sCysC as a predictor of mortality or early AKI biomarker among septic patients. Recently, in a large multicenter study (30), it was found that sCysC was less sensitive for AKI detection than SCr among postcardiac surgery patients.

For the mouse survival experiments, we chose to measure sCysC, SCr, and BUN at 6 h after the induction of sepsis because this is when the animals become clinically sick and, hence, in this model, mimics the time needed to diagnose sepsis in a clinical setting. Both sCysC and SCr did not show a very strong correlation with the time to death (with $R^2 < 0.7$ for both), as AKI is not expected to be the primary cause of sepsis mortality, and dysfunction of other organs/systems also contributes to mortality during sepsis.

**Conclusions.** We evaluated the ability of sCysC to monitor the progression of sepsis-AKI in the clinically relevant mouse model of polymicrobial sepsis in CD-1 mice. Among the three studied filtration biomarkers (sCysC, SCr, and BUN), sCysC showed a more robust increase early after sepsis. The blunted increase in sCysC after sepsis in BiNx, despite the increase in other nonrenal injury biomarkers, was caused by a sepsis-induced reduction of sCysC production and possibly the increase in SCr nonrenal elimination. Thus, sCysC is a better early detection biomarker than SCr in sepsis but is still influ-
enced by nonrenal factors that conspire to limit the accurate prediction of GFR during the evolution of sepsis-AKI. BUN levels after BiNx were not affected by sepsis but corresponded poorly to iGFR.

sCysC did not outperform SCr in identifying mice at risk of early or late mortality in this model of sepsis. sCysC can be used interchangeably with SCr for mortality risk stratification, and it increases more, early after the insult, and correlates better with iGFR than SCr.

sCysC may not be an ideal biomarker for sepsis-AKI, but because an early biomarker may enhance risk stratification and facilitate early diagnosis, sCysC may be, indeed, a better biomarker of sepsis-AKI than SCr. Although BUN also increased early after CLP, it did not correlate well with iGFR and was inferior to sCysC and SCr for classification of mortality risk.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES

