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A Furosemide Excretion Stress Test Predicts Mortality in Mice After Sepsis and Outperforms the Furosemide Stress Test During Vasopressin Administration

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Objectives: The furosemide stress test measures the volume of urine produced after a furosemide challenge. Furosemide stress test has previously demonstrated sensitive and specific prediction of progression to Kidney Disease: Improving Global Outcomes guideline defined acute kidney injury stage III in the ICU. Furosemide is actively excreted into the nephron lumen where it inhibits the sodium-potassium-chloride cotransporter, causing diuresis. We hypothesize that furosemide excretion is a more direct measure of tubule health than diuresis.

Design: We developed a furosemide excretion stress test to evaluate this hypothesis in a murine model of septic-acute kidney injury.

Setting: Basic science laboratory.

Subjects: Male and female 8-week old CD-1 mice.

Interventions: Sepsis was induced by cecal ligation and puncture in male and female mice. Furosemide stress test/furosemide excretion stress test started 42 hours post-cecal ligation and puncture with a 1 mg/kg furosemide bolus and urine was collected for 12 hours. The mice were then euthanized or monitored until 7 days post-cecal ligation and puncture. In another cohort, mice were treated with vasopressin, which decreases urine volume. Furosemide concentration was determined by high performance liquid chromatography.

Measurements and Main Results: Urine production during the 12-hour collection varied from 0.08 to 2.62 mL. Both urine production (furosemide stress test) and furosemide excretion (furosemide excretion stress test) predicted mortality (area under the receiver operating characteristic curve = 0.925 and 0.916) and time of death ($R^2 = 0.26$ and 0.74). Male and female mice demonstrated consistent results. Following vasopressin treatment, furosemide stress test specificity fell to 33% ($p = 0.016$) but furosemide excretion stress test specificity was maintained.

Conclusions: The furosemide stress test and furosemide excretion stress test performed similarly in predicting mortality; however, furosemide excretion stress test was superior in predicting time to death and maintained performance when challenged with vasopressin treatment in a mouse sepsis model.

Key Words: acute kidney injury; distal tubule; furosemide; proximal tubule; sepsis

Acute kidney injury (AKI) is associated with significant morbidity and mortality (1). Specific therapies and tools for predicting progression are currently lacking (2, 3). Improved accuracy in predicting progression would be useful both for management of individual patients and for conducting clinical trials to select patients at risk for progression (4). Traditional biomarkers such as serum creatinine and blood urea nitrogen (BUN) indirectly reflect glomerular filtration rate (GFR). Another class of biomarkers, including tissue inhibitor of metalloproteinases 2 and insulin-like growth factor-binding protein 7 are surrogates for kidney injury/damage (3, 5). A third type of biomarker has been recently developed that reflects the active response of the kidney to a stimulus, that is, a stress test: the furosemide stress test (FST) predicted clinically relevant outcomes (6) and FST has subsequently been shown to be significantly more predictive than almost all other candidate AKI biomarkers (7).

Furosemide strongly binds albumin (8) and therefore is not filtered by the glomerulus. Instead, it is actively excreted by proximal tubules involving organic anion transporters 1 and 3 (OAT1 and OAT3) (9). Once in the tubular lumen, furosemide interacts with thick ascending limb sodium-potassium-chloride cotransporter (NKCC2), and inhibits the reabsorption of sodium, resulting in natriuresis-induced

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diuresis. A furosemide-induced increase in urine volume can then be measured. In ICU patients, Chawla et al (6) identified a cutoff value of 200 mL of urine produced within 2 hours following challenge as giving the best combination of specificity and sensitivity to predict progression to Kidney Disease: Improving Global Outcomes guideline defined AKI stage III within 14 days.

The proximal tubules are an important site of damage and mediator of outcomes in AKI (10–12). The health of proximal tubules (site of furosemide secretion) will be the largest determinant of urine volume during FST. Smaller effects from interactions with the GFR and other regions of the kidney are also likely (13). We hypothesized that furosemide excretion, namely the fraction of the furosemide dose administered that is recovered in the urine (called furosemide excretion stress test [FEST]), would be a more specific indicator of proximal tubule health. To test this, we developed an HPLC assay to measure urinary furosemide concentration and developed versions of FST and FEST suitable for use in mice. We began by comparing FST and FEST in a murine model of sepsis. We then used vasopressin, which is used for hemodynamic support, to investigate the robustness of each test in a clinically relevant situation.

MATERIALS AND METHODS

Detailed methods are reported in the **Supplemental Methods** (Supplemental Digital Content 1, http://links.lww.com/CCX/A162). Briefly, an Animal Care and Use Committee-approved, standard mouse cecal ligation and puncture (CLP) model, with fluid and antibiotic treatment was used (14). In some mice, 0.069 U/hr/kg vasopressin was administered intraperitoneally by osmotic minipump. At 42 hours, mice were given 1 mg/kg furosemide and urine was collected for 12 hours in metabolic cages. Mice were either euthanized for analysis of blood and kidney contents or monitored in a survival study (**Fig. 1**). We developed a HPLC assay to quantitate urine furosemide.

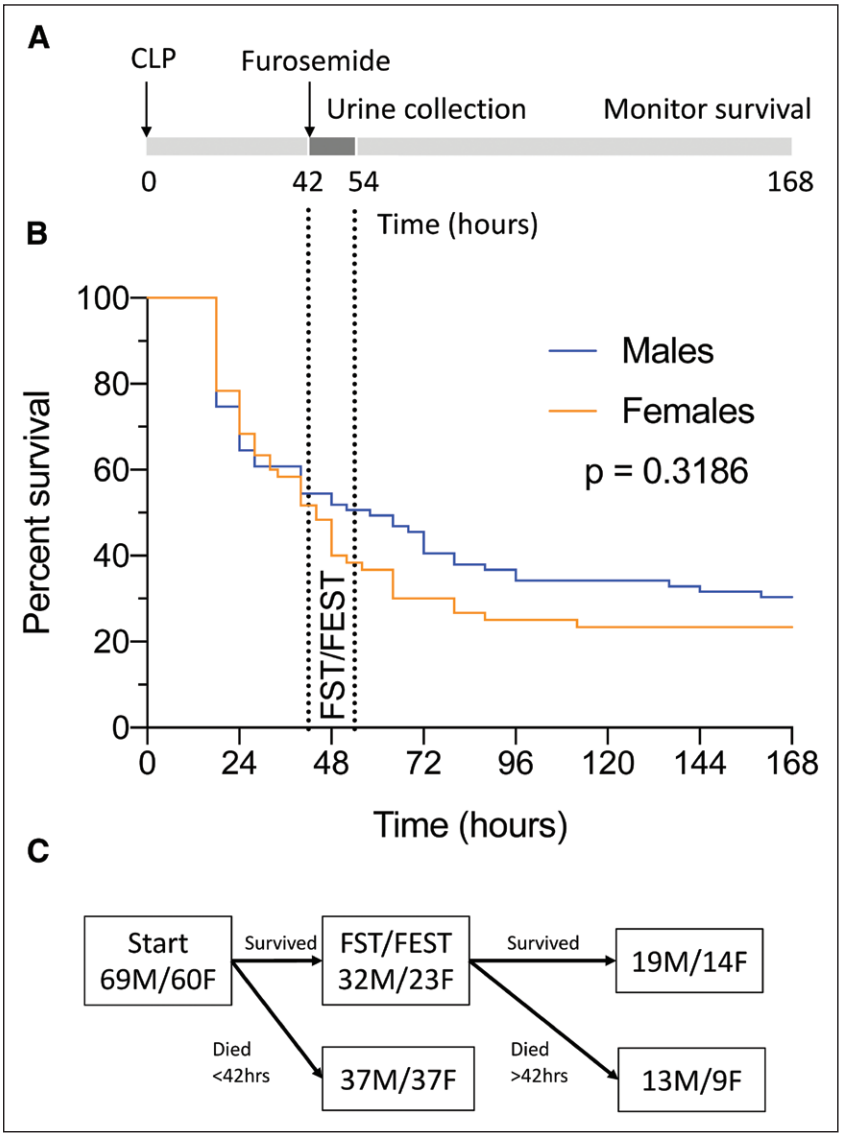

Figure 1. Experimental summary. **A**, Experimental design showing timing of furosemide treatment and urine collection in relation to cecal ligation and puncture (CLP). **B**, Overview of observed survival following CLP in the male and female cohorts. **C**, Flowchart illustrating outcomes in the male and female cohorts. FEST = furosemide excretion stress test, FST = furosemide stress test.

RESULTS

The Furosemide Stress Test Can Be Adapted for Mouse Models

A protocol for conducting the FST in an animal model would enable the study of aspects of the test that would be challenging or impossible to assess in the clinical setting. To realize this objective, we modified the original FST protocol as follows: rather than inserting a Foley catheter and monitoring under anesthesia, we chose to use conscious mice and rely on their normal urination. To determine the appropriate collection time, urine was collected from 0 to 6 hours and from 6 to 12 hours post furosemide injection.

In nine of 11 normal mice almost all furosemide was in the 0–6 hour fraction. In the remaining two mice, 6% and 32% was recovered during the 0–6 hour fraction with the remainder in the 6–12 hour fraction (**Supplementary Fig. 1**, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). To maximize predictive performance a 12-hour collection period was used.

Furosemide Excretion and Urine Production Predict Mortality in a Murine Model of Sepsis

To model ICU patients from the clinical study (6) in mice, we chose the sepsis model of CLP surgery treated with fluids and antibiotics. Following CLP surgery, bacterial peritonitis occurs with a rapid inflammatory response and subsequent organ injury, including AKI. We administered furosemide with fluids and antibiotics on the second morning following surgery ($t = 42$ hr; Fig. 1), attempting to simulate the timing of FST used in a patient with uncertain prognosis, while also minimizing stress on the animals. In a cohort of 32 male mice that survived to $t = 42$ hours, the urine volume

produced after furosemide challenge varied from 0 to 2.62 mL and was higher in the mice that survived to the end of the study ($p < 0.0001$; **Fig. 2*A***). Furosemide-stimulated urine production predicted 7-day survival, with an area under the receiver operating characteristic curve (AUC ROC) of 0.919 ($p < 0.0001$; **Fig. 2*B***). Similarly, the percentage of furosemide recovered in the urine was higher in mice that survived ($p < 0.0001$; **Fig. 2*D***). The percentage of furosemide recovered in the urine predicted survival with an AUC ROC of 0.875 ($p = 0.0004$; **Fig. 2*E***).

In addition to predicting mortality, FEST correlated with, and could predict, time of death in nonsurvivors ($R^2 = 0.751$; $p = 0.0001$; **Fig. 2*F***). The correlation between FST and time of death did not reach statistical significance ($R^2 = 0.297$; $p = 0.0540$; **Fig. 2*C***)

FST and FEST Weakly Correlate With Existing Markers of Kidney Injury, Messenger RNA, and Cytokines

To identify potential mediators or confounders of FST and FEST in the mouse model, CLP surgery was performed in a second cohort of male mice. At the end of the urine collection period ($t = 54$ hr post-surgery) this cohort was euthanized and serum and tissue collected.

Consistent with these tests being indicators for kidney health, the response in both FST and FEST weakly correlated with BUN measured at 54 hours (FST: $R^2 = 0.335$, $p = 0.0302$; FEST: $R^2 = 0.382$, $p = 0.0185$; **Fig. 3**, ***A*** and ***F***). A similar correlation was also observed with the liver injury marker alanine transaminase (FST: $R^2 = 0.378$, $p = 0.0194$; FEST: $R^2 = 0.437$, $p = 0.0101$; **Supplementary Fig. 2, *B*** and ***E***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162) but not with aspartate transaminase (data not shown). Creatine kinase also correlated with FEST ($R^2 = 0.3033$; $p = 0.0412$; **Fig. 3*H***). No correlation was observed with lactate dehydrogenase, amylase, or alkaline phosphatase (data not shown).

FST and FEST did not correlate with kidney tissue expression of OAT1 nor OAT3, the two main transporters for furosemide (data not shown). Lower expression of NKCC2, the target for furosemide, was observed in mice with low FST and FEST responses (FST: $R^2 = 0.333$, $p = 0.0307$; FEST: $R^2 = 0.458$, $p = 0.0079$; **Fig. 3, *A*** and ***C***).

Pro- and anti-inflammatory cytokines can be synthesized by kidneys, including interleukin (IL)–6 which has been shown to predict mortality (15, 16). Higher kidney expression of IL-6 was observed in mice with low FST and FEST responses (FST: $R^2 = 0.239$, $p = 0.0770$; FEST: $R^2 = 0.354$, $p = 0.0247$; **Fig. 3, *B*** and ***D***). There was no correlation with IL-1β, tumor necrosis factor alpha, or IL-10 (data not shown).

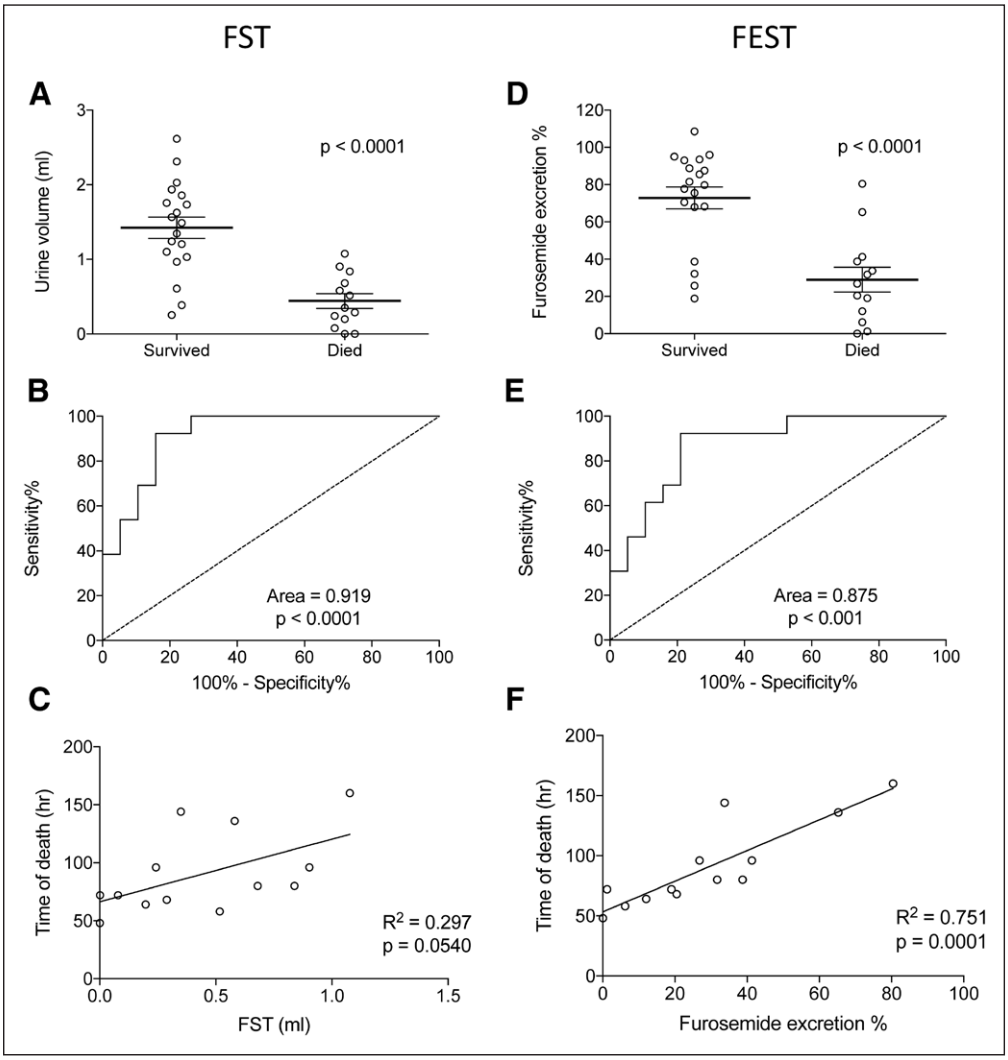

Figure 2. Performance of furosemide stress test (FST) and furosemide excretion stress test (FEST) during sepsis in male mice. FST: **A**, Comparison of urine volume following furosemide bolus in mice that survived and died. **B**, Receiver operator characteristic curve for FST. **C**, Plot of urine volume against time of death. FEST: **D**, Comparison of furosemide excretion following furosemide bolus in mice that survived and died. **E**, Receiver operator characteristic curve for FEST. **F**, Plot of furosemide excretion against time of death.

Assessment of FST and FEST in a Female Cohort

To test whether these results were broadly applicable, we repeated the study in female mice. The urine volume produced after furosemide challenge varied from 0.19 to 2.73 mL (**Supplementary Fig. 3*A***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). As was observed in males, urine volume was higher in the mice that survived to the end of the study ($p = 0.0008$). Urine production was predictive of survival and comparable to the males (AUC ROC = 0.948; $p = 0.0004$; **Supplementary Fig. 3*B***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). There was perfect separation in furosemide excretion in the females between survivors and nonsurvivors ($p < 0.0001$; **Supplementary Fig. 3*D***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). FEST therefore performed better than FST in this cohort (AUC ROC = 1.0;

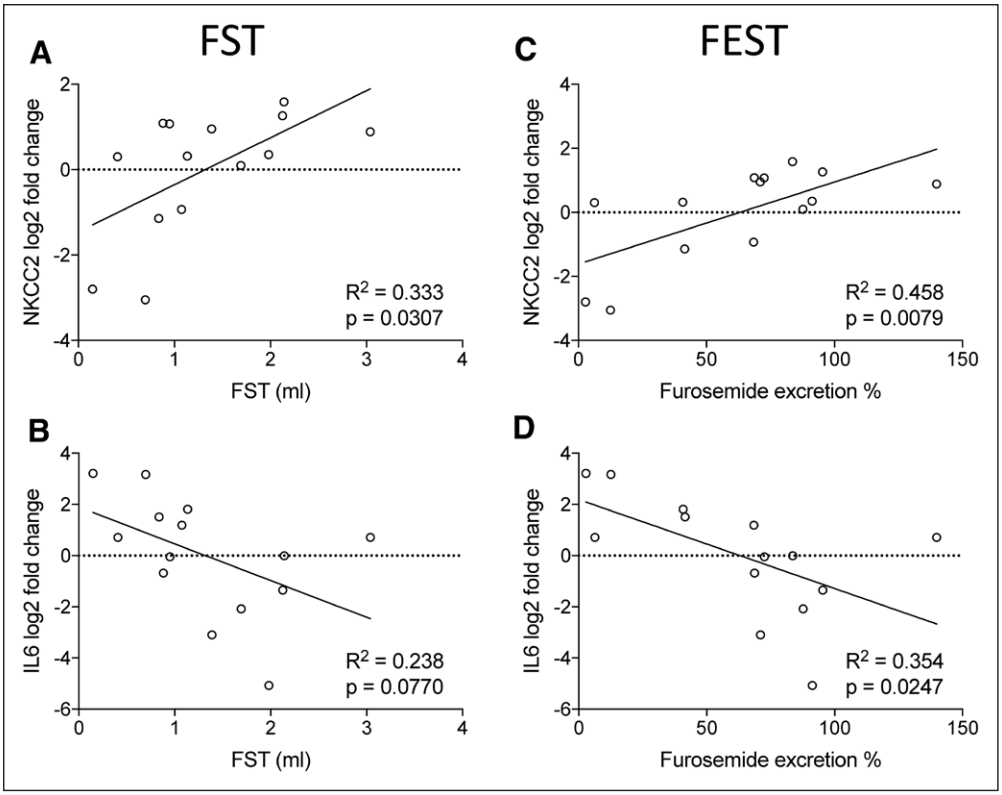

Figure 3. Comparison of urine volume (furosemide stress test [FST]) and furosemide excretion (furosemide excretion stress test [FEST]) with kidney tissue messenger RNA (mRNA) expression. FST and FEST correlations with kidney sodium-potassium chloride cotransporter (NKCC2) mRNA (**A** and **C**, respectively), and kidney interleukin-6 (IL-6) mRNA (**B** and **D**).

$p = 0.0001$; **Supplementary Fig. 3*E***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162).

In contrast to the males, the correlation between time of mortality and FST or FEST in the females was weaker ($R^2 = 0.152$; $p = 0.3001$ and $R^2 = 0.233$; $p = 0.2259$, respectively [**Supplementary Fig. 3**, ***C*** and ***F***, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). This may be due to a narrower distribution in time of mortality in the female cohort compared with the male cohort (Fig. 1*B*).

FST and FEST correlated strongly in the aggregated male and female dataset ($R^2 = 0.662$; $p < 0.0001$; **Supplementary Fig. 4**, Supplemental Digital Content 1, http://links.lww.com/CCX/A162). Consistent with the individual tests, the survivors and nonsurvivors clustered separately and no differences were evident between the males and females.

Combining the male and female experiments had comparable performance to analyzing them individually (**Fig. 4**). Both urine volume and furosemide excretion were higher in the mice that survived to the end of the study ($p < 0.0001$; **Fig. 4*A*** and ***D***). The AUC ROC for FST was 0.925 ($p < 0.0001$; **Fig. 4*B***) and for FEST was 0.916 ($p < 0.0001$; **Fig. 4*E***)). The correlation observed in the males for time to death was preserved in the combined group for both FST ($R^2 = 0.258$; $p = 0.0159$; **Fig. 4*C***) and FEST ($R^2 = 0.742$; $p < 0.0001$; **Fig. 4*F***). For FST, performance was maximized at a cutoff of 0.94 mL with sensitivity of 91.6% and specificity of 78.8%. For FEST, a cutoff of 44% achieved 91.3% sensitivity and 78.8% specificity.

Assessment of FST and FEST During Vasopressin Administration

To further explore the clinical utility of these two tests, we applied them in a group of mice receiving vasopressin. In the context of sepsis, vasopressin is commonly used as a vasopressor in septic patients (17, 18) but can increase or decrease urine output in any given patient, which may interfere with the response to furosemide.

Vasopressin did not alter mortality compared with the control water-treated group or previous experiments (**Fig. 5*A***). In a control water-treated group urine production was consistent with previous experiments (1.09 mL) but decreased (0.57 mL; $p = 0.0490$) with vasopressin treatment (**Fig. 5*B***). There was no statistically significant change in furosemide excretion ($p = 0.1291$; **Fig. 5*C***).

If the FST and FEST cutoffs from the previous experiment are robust, applying them to predict outcomes in the control water-treated and vasopressin-treated groups from this experiment should result in similar sensitivity and specificity (null hypothesis). This was evaluated by constructing a contingency table of true negatives and false positives (**Supplementary Table 1**, Supplemental Digital Content 1, http://links.lww.com/CCX/A162) and applying Fisher exact test. Comparing the control water-treated group to the earlier experiments gave a similar fraction of true negatives and false positives for both FST ($p = 0.6446$) and FEST ($p > 0.99$). In contrast, comparing the vasopressin-treated group to the earlier experiments FST and FEST responded differently. For FST, the reduced urine production in the vasopressin-treated group more than halved specificity (33%), altering the proportion of true negatives, as well as false positives ($p = 0.0157$). The performance of FEST was relatively unaffected by vasopressin treatment, consistent with the previous experiment ($p = 0.2086$).

DISCUSSION

The FST has previously been demonstrated to have superior predictive value for prognosis of AKI, including progression of AKI and mortality in the ICU (6, 7). Because FST is tightly linked to overall kidney function (requiring proximal tubule secretion and thick ascending limb contribution), FST can predict AKI and AKI progression, which has direct clinical value to guide renal replacement therapy, and FST is robust enough to predict mortality, despite contributions from several nonrenal factors. In this study, we have built on these findings by investigating a more direct measure of proximal tubule health, the FEST, that directly measures the fraction of

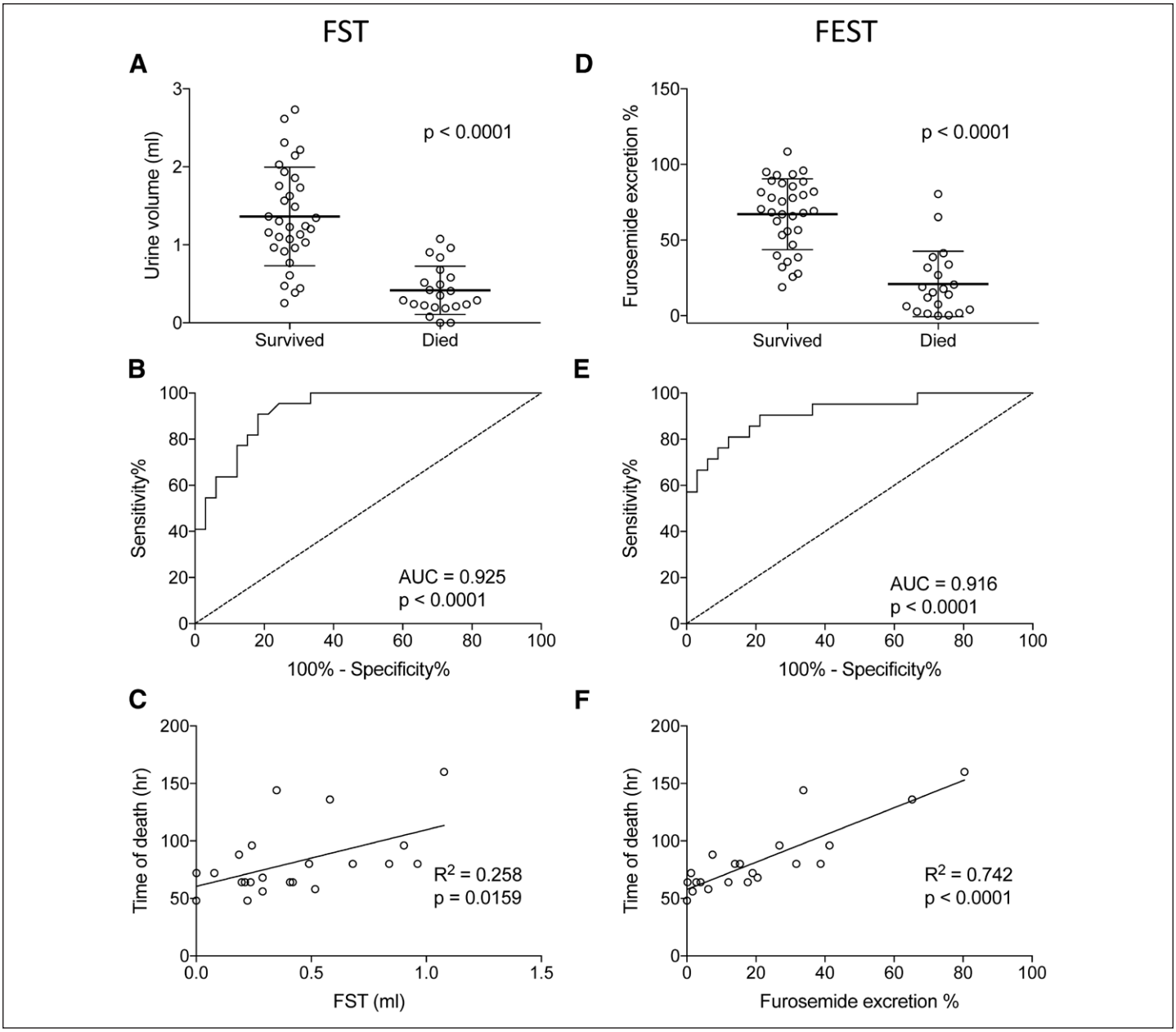

Figure 4. Combined analysis of performance of furosemide stress test (FST) and furosemide excretion stress test (FEST) during sepsis in both male and female mice, with data pooled from Figure 3 and Supplementary Figure 2 (Supplemental Digital Content 1, http://links.lww.com/CCX/A162). FST: **A**, Comparison of urine volume following furosemide bolus in mice that survived and died. **B**, Receiver operator characteristic curve for FST. **C**, Plot of urine volume against time of death. FEST: **D**, Comparison of furosemide excretion following furosemide bolus in mice that survived and died. **E**, Receiver operator characteristic curve for FEST. **F**, Plot of furosemide excretion against time of death. AUC = area under the curve.

administered furosemide recovered in the urine. Specifically, we 1) developed a method to quantitate urinary furosemide, 2) developed a version of FST and FEST for murine models, and 3) explored the capabilities and limitations of both tests. Although FST and FEST initially performed similarly, only FEST maintained performance during vasopressin-treated sepsis in our mouse model.

The Rationale for the Furosemide Excretion Stress Test

The FEST was created after considering the proposed mechanism for the FST. Because FST (6, 7) relies on a connected series of steps, we hypothesized that furosemide excretion, as a more direct measurement, may provide better performance. Furosemide in the circulation is bound to albumin and is not freely filtered at the glomerulus (8). Instead, furosemide is actively secreted into the proximal tubule lumen by OATs. Furosemide then binds NKCC2 in the thick ascending limb and reduces its activity. By inhibiting the reabsorption of sodium diuresis ensues (19). The relationship between outcome and the FST therefore depends on the GFR, varies with the state of both the proximal tubules and thick ascending limb, and can be confounded by agents that affect water reabsorption in the collecting duct (20, 21). By measuring the excretion of furosemide, the FEST should more directly focus on the functional status

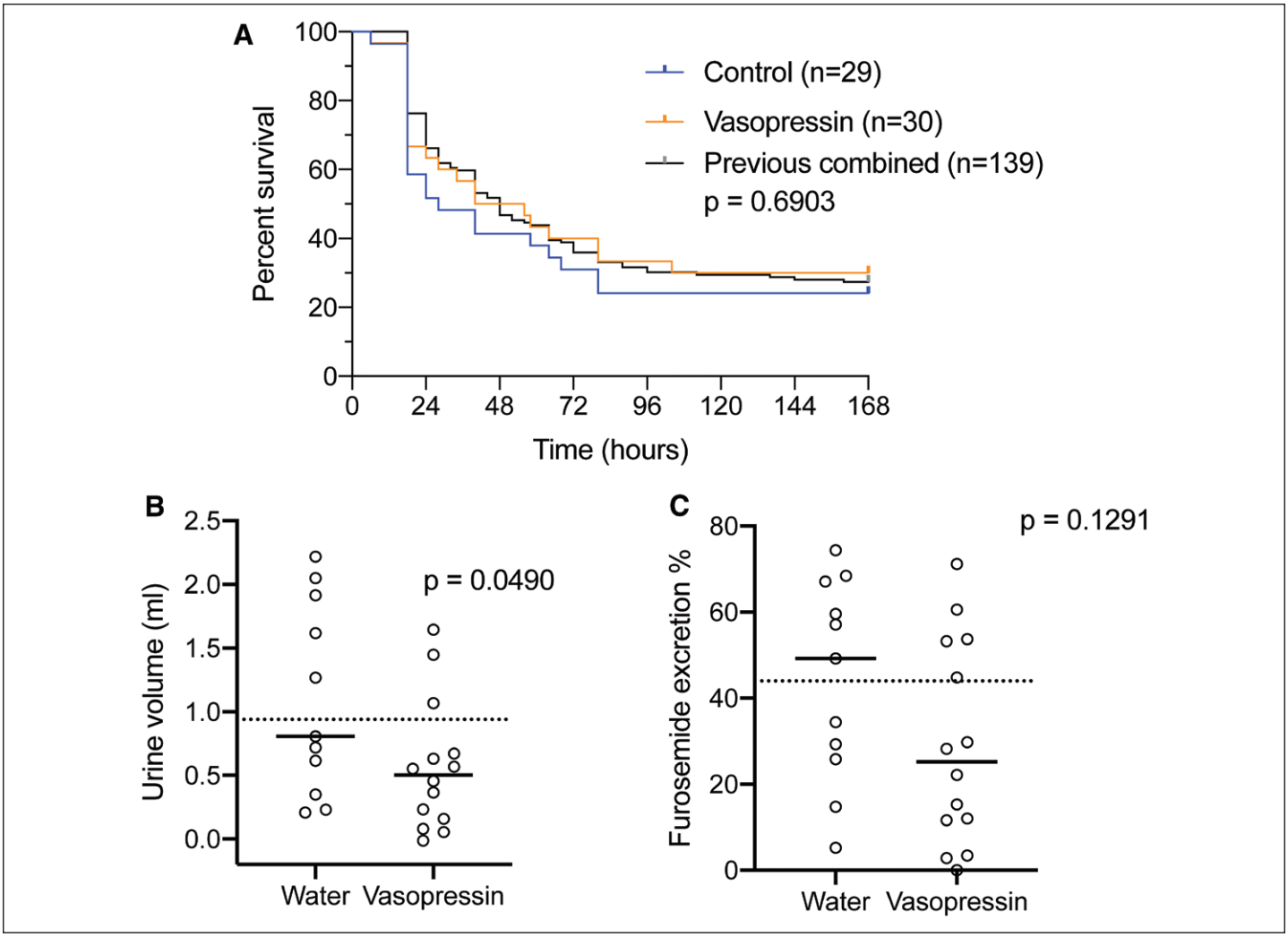

Figure 5. Effect of vasopressin treatment on furosemide stress test (FST) and furosemide excretion stress test (FEST) performance. **A**, Comparison of survival in the control water-treated and vasopressin-treated groups with the combined male and female experiments from Figure 4. Twenty-five (11 control water-treated, 14 vasopressin) mice survived to 42 hr to receive FST/FEST. Of these mice, 16 survived (7/9) to 7 d. Effect of vasopressin treatment on urine volume (**B**, FST) and furosemide excretion (**C**, FEST). The cutoff values giving optimal performance in the combined male and female cohort are indicated by *dashed lines*.

of proximal tubules, while avoiding some of the potential confounding factors that can influence water excretion.

Applying FST and FEST in Mice

Some adjustments were made in how the tests were performed in the mice compared with humans. A longer collection time was used to avoid requiring catheterization. Although the possible collection of urine after the furosemide-induced diuresis may weaken FST performance, oliguria and anuria are commonly observed in sepsis-AKI, which would minimize this confounding variable. An advantage of the FEST in mice is that it should be insensitive to how much urine was produced before and after the dose of furosemide clears. The same weight-adjusted dose of furosemide was used in this study in mice as was previously used in the human studies (6). The route of injection was changed from IV to subcutaneous to minimize stress in the mice.

Performance of FST and FEST

The robust performance of the FST in the initial clinical study has prompted several further studies in an expanded range of conditions including critical illness (22), cardiopulmonary bypass surgery in infants (23), need for renal replacement therapy in AKI (24), early graft function in kidney transplantation (clinical trials identifier NCT03071536), and chronic kidney disease (NCT02417883). To explore the nuances of FST and the relative advantages of FST and FEST, we applied them in a sepsis-AKI mouse model.

Due to the previous observation of vacuolization (25) in proximal tubular epithelial cells, we anticipated proximal tubule dysfunction to occur in this mouse sepsis model (10–12). The renal and nonrenal outcomes of the mice in this study were highly variable, with FST and FEST values reflecting this variability. Consistent with observations from the human studies, FST did not correlate strongly with other biomarkers (7), nor did FEST.

Evaluating FST and FEST in mice facilitated comparisons, such as tissue gene expression, that would be challenging to assess in humans. Neither OAT1 nor OAT3 gene expression in the kidney correlated with FST or FEST, consistent with regulation of furosemide transport at the posttranslational or metabolic level. Expression of the target for furosemide, NKCC2, did correlate

with FST and may suggest thick ascending limb dysfunction also occurs in this model.

FST strongly predicted mortality in mice with performance comparable to the previous human study (6). FEST, although equivalent, performed no better than FST in predicting mortality in our initial study. Although we hypothesized that differences in GFR and damage to other nephron segments might compromise FST performance more so than FEST performance, differences in these factors will be minimized in our CLP model. All mice were subjected to the same injury, at the same time, and received the same fluids and antibiotics. The severity of injury remains a source of variability altering mortality and time of death. It is notable therefore that time of death was more strongly predicted by FEST. In the patient population, causes of sepsis in humans and treatments are more varied than in CLP, so we next looked at performance in different experimental designs.

Performance Assessment

The expression of OATs display significant inter-individual variation (26). Sex differences are also observed in some species including rats (27). Although the robust performance, despite inter-individual variation, suggests that FST and FEST performance would not display sex differences, the survival study was repeated in a female cohort. Both FST and FEST were able to predict mortality with performance comparable to that observed in the male cohort. Mortality in the female cohort occurred over a narrower time period, weakening the correlation with time of mortality compared with the males. However, combining the male and female cohorts performed comparably to the male cohort alone, suggesting a similar underlying relationship between response to furosemide and propensity toward mortality.

Based on the results from the male and female cohorts, we identified cutoffs for FST and FEST that maximized sensitivity and specificity. We applied these cutoffs to a modified model that included vasopressin treatment, intended to reflect the heterogeneity in human patients. The reduced urine production with vasopressin treatment in mice improved the already high FST sensitivity but dramatically decreased specificity. Vasopressin can at least transiently increase urine output and creatinine clearance in some patients (28, 29), which we speculate may be due to an interplay between improvement in blood pressure and hence GFR, and the antidiuretic effects of vasopressin. Because this interplay may have a different net effect in any individual patient, tests such as FST and FEST need to be validated in vasopressin-treated sepsis patients, and the multiple and potentially opposite effects of vasopressin on urine output may decrease the performance of FST, whereas FEST should be relatively unaffected by vasopressin treatment.

Challenges to Clinical Utility

Clinical value of a biomarker requires not only performance, but also timeliness. In addition to the steps required for the FST to collect and measure the volume of urine produced, FEST adds the additional requirement of measuring the concentration of furosemide. In this study, quantification was performed by HPLC. Point of care devices have been developed for some drugs (30, 31) where HPLC might otherwise be used and similar development for furosemide could shorten the analytic time.

CONCLUSIONS

We have adapted FST to a mouse model and developed the complementary FEST. Both FST and FEST have demonstrated similar capacity to predict mortality with FEST more accurately predicting time of death in the mice that died. Although FST is simpler to implement, the superiority of FEST in predicting time of death and robustness to vasopressin treatment warrants its continued evaluation in ongoing and future trials of FST in the ICU and other settings.

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